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(54) Title: GLYCOSYLATION DEFICIENT SIV AND HIV ENVELOPE GLYCOPROTEINS


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SIVmac239   114 NKSETDRWGLTKSITTTASTTSTTASAKVDMVNETSSCIAQDNCT   158
g4              Q...........................................
g5              .......................................Q...
g6              .......................................Q..
g45             Q......................................Q..
g46             Q......................................Q..
g56             ...................................Q.....Q..
g456            Q..................................Q.....Q..
M144V           ..............................V............
g456MV          Q.............................V.Q.......Q..
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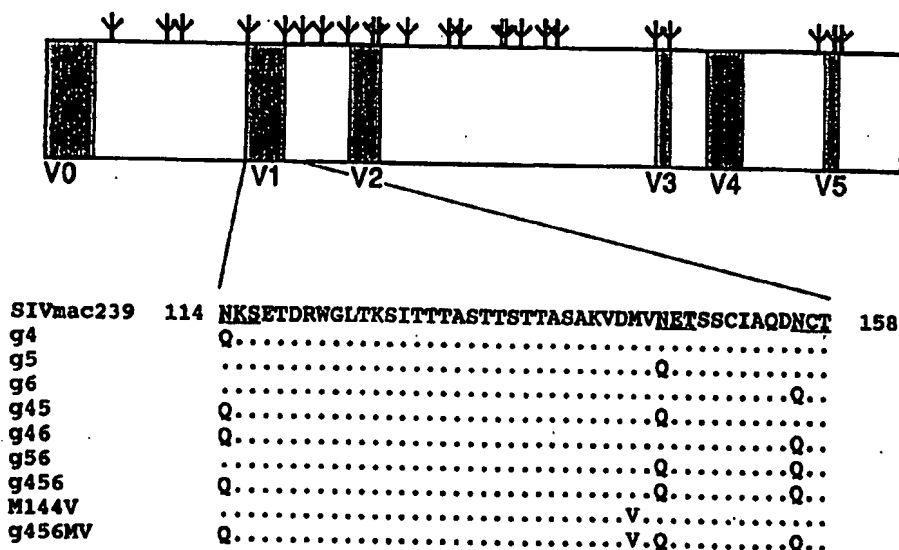
(57) Abstract

The present invention is based on the discovery that selectively removing N-linked glycans within the amino-terminal portion of a recombinant gp120 glycoprotein of immunodeficiency viruses such as human immunodeficiency type I or simian immunodeficiency virus produces a selectively underglycosylated envelope glycoprotein capable of enhanced antibody responses.

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GLYCOSYLATION DEFICIENT
SIV AND HIV ENVELOPE GLYCOPROTEINS

BACKGROUND OF THE INVENTION

1.   Field of the Invention

Embodiments of the present invention relate to the human immunodeficiency virus and

10   vaccines therefor. More particularly, embodiments of the present invention relate to selectively

underglycosylated envelope glycoproteins useful as HIV-1 vaccines.

2.   Description of Related Art

Human immunodeficiency virus (HIV) is the etiological agent of acquired immune

15   deficiency syndrome (AIDS). The envelope (env) gene of HIV encodes a 160 kilodalton

glycoprotein which is cleaved into an extracellular protein known as gp120 and a transmembrane

protein known as gp41. Among HIV-1 isolates, the envelope glycoproteins contain conserved

cysteine residues and N-linked carbohydrate sites. The gp120 molecule contains 5 variable

regions referred to as V1 - V5. These variable regions are designated as such because they exhibit

20   amino acid sequence variability across HIV-1 isolates. Gp120 also contains constant regions, i.e.,

regions of relatively conserved amino acid sequence across HIV-1 isolates.

The HIV envelope protein gp120 is heavily glycosylated, having about 55% of its

molecular mass contributed by N-linked carbohydrates. HIV-1 molecular clones contain an

average of 23-24 potential N-linked glycosylation sites on gp120. Carbohydrate side chains of

envelope glycoproteins of HIV-1 and other viruses have been postulated to interfere with binding

of neutralizing antibodies. To date, however, it has not been demonstrated that the absence of

glycosylation sites enhances the antibody response to gp120.

    Back et al., 1994, *Virology* 199:431; Botarelli et al., 1991, *J. Immunol.* 147:3128; Doe

5   et al., 1994, *Eur. J. Immunol.* 24:2369; Bolmstedt et al., 1992, *J. Gen. Virol.* 13:3099; and PCT

US93/17705 teach that selective deglycosylation of carboxy-terminal sites in HIV-1 gp120 may

be associated with increased antigenicity of the resultant molecule, as determined via *in vitro* CTL

response or antibody binding. PCT US93/17705 is said to discover that selectively deglycosylated

HIV-1 envelop proteins retain their ability to support viral infectivity, and note that the envelope

10   protein of the related simian virus for African Green Monkeys, which is not pathogenic to its

natural host, has fewer N-linked glycosylation sites, particularly in the C-terminal portion of the

analogous gp120. PCT US93/17705 teaches that the position of deglycosylation in gp120 should

be between the C-terminus of gp120 and the Cys residue at the N-terminal side of the cysteine

loop containing the hypervariable region 3 (V3) (i.e., at about position 296, the C-terminal end

15   being about amino acid 480). The carboxy terminal sites of glycosylation which have been

focussed on in the literature include the region encompassed by the N-terminal boundary of

variable region 3 (V3) (i.e., amino acid 296) to the carboxy-terminal end of the molecule,

including sites at about 386, 392, 397, 406, 463, and in some cases, 448 and/or 392. Such sites

are deglycosylated by mutating the natural DNA sequence such that the consensus N-linked

20   glycosylation sequence is altered, e.g., via substitution of Asn, Ser or Thr with a different amino

acid. The consensus sequence of the site for N-linked glycosylation is Asn-X-Ser/Thr, where X

is any amino acid except Pro and Asp.

    Lee et al., 1992, *Proc. Nat. Aca. Sci.* 89:2213, and Wang et al., 1996, *J. Virol.* 70:607,

examine the relative importance of each of the 24 N-linked glycosylation sites individually of

gp120 to viral infectivity. It was determined that the N-terminal half of gp120 contained N-linked

glycosylation sites which are necessary to maintain viral infectivity. The five consensus N-linked

glycosylation sites that are likely to have important roles in infectivity were determined to be

located in the N-terminal half of gp120. Representative sites were identified as 88, 90, 141, 143,

5   197, 199, 262, 264, 276 and 278. Lee et al. suggest that N-linked glycosylation sites located in

the C-terminal half of gp120 are more dispensable for viral infectivity than those located in the

amino-terminal half, and suggest that HIV-1 vaccine candidates would comprise those selectively

deglycosylated gp120 molecules which maintained the biological activity leading to virus

infectivity.

10   Gram et al., 1994, *Arch. Virol.* 139:253, identify an N-linked glycan in the V1-loop of

HIV-1 gp120 which did not appear to affect infectivity of a virus containing the gp120 mutant in

cell culture but which appeared to render the virus more resistant to neutralization by monoclonal

antibodies to the V3-loop and neutralization by soluble recombinant CD4.

15   **BRIEF SUMMARY OF THE INVENTION**

Embodiments of the present invention are based on the discovery that selectively removing

N-linked glycans within the amino-terminal portion of a recombinant gp120 glycoprotein of

immunodeficiency viruses such as human immunodeficiency virus type 1 (HIV-1) and simian

immunodeficiency virus (SIV) produces a selectively underglycosylated envelope glycoprotein

20   capable of enhanced antibody responses useful as an HIV-1 vaccine.

According to a certain embodiment of the present invention, a compound is prepared

which includes a recombinant human immunodeficiency virus type 1 envelope glycoprotein having

an amino acid sequence which is altered with respect to a wild type HIV-1 envelope glycoprotein.

The altered amino acid sequence includes a mutated consensus amino acid recognition sequence

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for N-linked carbohydrate attachment which, as a result of the mutated consensus amino acid recognition sequence, is not glycosylated in a mammalian host cell. The resulting amino acid is referred to herein as being underglycosylated. The mutated consensus amino acid recognition sequence is positioned between the N-terminus of gp120 and the Cysteine at the N-terminal side of the gp120 cysteine V3 loop. The Cysteine is approximately at amino acid position 296. In addition, the recombinant envelope glycoprotein has a mutated or otherwise altered consensus amino acid recognition sequence for N-linked carbohydrate attachment and is infective, i.e., when present as a component of a complete HIV virion, it supports viral infectivity.

Additional embodiments of the present invention are directed to pharmaceutical compositions and/or vaccines (both for protecting uninfected individuals or for treating infected individuals) that comprise such HIV-1 recombinant envelope proteins having altered sequences as described herein in pharmaceutically acceptable carriers or excipients. Methods including administering such pharmaceutical compositions or vaccines to humans to stimulate the production of antibodies against HIV are also contemplated.

Still other embodiments of the present invention include DNA encoding the HIV-1 recombinant envelope proteins having altered sequences as described herein (particularly in an expression vector), recombinant cells comprising such DNA, and methods of making the recombinant mutant envelope glycoproteins by expressing such DNA. Methods according to the present invention include delivering such DNA to cells to produce a translation polypeptide immunizing product capable of delivering an immune response. The methods of the invention may be applied by direct injection of the DNA into cells of an animal, including a human, in vivo, or by in vitro transfection of some of the animal cells which are then reintroduced into the animal body. The DNA may be delivered to various cells of the animal body, including muscle, skin, brain, lung, liver, spleen, or to cells of the blood. Delivery of the DNA directly in vivo is

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preferable to the cells of muscle or skin. The DNA may be injected into muscle or skin using an injection syringe. The DNA may also be delivered into muscle or skin using a vaccine gun.

Still further embodiments of the present invention include antibodies raised against, or preferentially binding to, the mutant envelope glycoprotein.

5   Other objects, features and advantages of embodiments of the present invention will become more fully apparent from the following description taken in conjunction with the accompanying drawings and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

10   In the course of the detailed description of certain preferred embodiments to follow, reference will be made to the attached drawings in which,

Fig. 1 is a schematic illustration of the HIV-1 envelope glycoprotein gp120, with the hypervariable regions of the molecule indicated by darkened lines, designated V1-V5, wherein cysteine-cysteine disulfide bonds are represented by solid lines connecting each end of a loop.

15   Numbers represent the first amino acid in each of the 24 potential N-linked glycosylation sites in the molecule.

Fig. 2 is a schematic illustration of gp120 from HIV-1, showing the distribution and amount of conservation of N-linked glycosylation sites. Amino acids are numbered from the N-terminus of the molecule to the C-terminus. The numbers beneath the diagram denote the

20   position of the first amino acid in the consensus sequence of an N-linked glycosylation site. Sites which are >90% conserved among HIV-1, HIV-2 and SIV isolates are indicated by an arrow with an open head and are numbered sequentially with the prefix 'b'. Other sites which are conserved at a level of less than 50% are indicated by an arrow having a wavy tail.

Fig. 3 is a schematic representation of the location of the glycosylation sites in SIVmac239

(identified by the tree symbol at the top of the figure) and particularly, the 4th, 5th, and 6th

glycosylation sites containing the consensus sequence Asn X Ser/Thr in the highly variable region

1 (V1) of the gp120 sequence of SIVmac239 that were selected for mutagenesis.   All seven

5   possible mutant forms of these sites were created and are referred to as g4, g5, g6, g45, g46, g56,

and g456.

Fig. 4 is a graph showing the rate of virus production of CEMx174 cells by SIV239mac

mutant viruses with single glycosylation substitutions in gp120.

Fig. 5 is a graph showing the rate of virus production of CEMx174 cells by SIV239mac

10   mutant viruses with multiple glycosylation substitutions in gp120.

Fig 6 is a graph showing rate of viral replication in Rhesus periferal blood mononuclear

cells (PBMC's).

Fig. 7 is a graph showing rate of replication of SIV glycosylation mutant g456 following

transfection of CEMx174 cells.

15   Fig. 8 is a graph showing rate of viral replication following infection of CEMx174 cells

with uncloned virus stock from g456 transfection.

Fig. 9 is a schematic showing the sequence of g456 revertant clones.

Fig. 10 is a graph showing rate of viral replication following infection of CEMx174 cells.

Fig. 11 is a graph showing rate of viral replication following infection of Rhesus monkey

20   cell line 221.

Fig. 12 is a sample of a gel electrophoresis showing migration of gp160 precursor and

gp120 external surface subunit from wild type and g456 mutant viruses.

Fig. 13 is a graph showing rate of virus production of CEMx174 cells by SIV239mac

mutant viruses with five glycosylation substitutions in gp 120.

Fig. 14 is a graph showing the results of an ELISA assay in which serum from monkeys

immunized with a replication competant SIV containing a recombinant gp120 protein having an

altered amino acid sequence according to the invention or with a wild type SIV virion by was

tested for the presence of antibodies able to bind to a peptide having the amino acid sequence

5   NH2- Cys Asn Lys Ser Gle Thr Asp Arg Trp Gly Leu -COOH (Peptide A) containing the altered

SIV gp120 sequence. Animals 344-95 and 346-95 were inoculated with the g45 virus. Animals

347-95 and 240-95 were inoculated with the g46 virus. Animals 245-95 and 252-95 received the

g56 virus. Animals 258-95 and 259-95 served as the control animals and received the wild-type

SIVmac239 virus.

10   Fig 15 is a schematic representation of the amino acid sequence of SIVmac239 (residues

89-213) with twenty-three peptides corresponding to the underlined sequences to be used in

determining which regions of the SIV envelope protein can elicit antibodies in infected animals.

Figs. 16-21 are graphs showing the immune response following infection for 16 weeks for

each peptide for the indicated virus.

15   Fig. 22 is a graph showing animal sera antibody responses to peptide 14 following 24

weeks infection with mutant and wild-type SIVmac239 viruses.

DETAILED DESCRIPTION OF CERTAIN PREFERRED EMBODIMENTS

Carbohydrates comprise about 50% of the mass of gp120, the external envelope

20   glycoprotein of the simian and human immunodeficiency viruses (SIV and HIV). When the

envelope precursor of gp120 is produced in mammalian cells in the presence of glycosylation

inhibitors, the protein generally is not properly processed. Deficits imparted by lack of

glycosylation include lack of proper folding, retention in the golgi, lack of proteolytic processing,

and inability to bind to CD4. When fully glycosylated gp120 is deglycosylated enzymatically in

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the absence of detergents, the deglycosylated gp120 apparently retains its native structure and can

bind CD4. Thus, carbohydrates appear to be required to generate a properly folded, properly

processed protein, but once formed the carbohydrates do not appear to be required to maintain

native structure. Despite this general requirement for carbohydrates, it has previously been shown

5   that individual N-linked sites can be eliminated without impairing native structure or the ability

of virus to replicate. However, it also has been shown that other N-linked sites are essential for

the virus to replicate.

Since the extensive glycosylation of HIV and SIV envelope proteins was initially

recognized, it has been speculated that the carbohydrates may form a barrier that can limit the

10   humoral immune response and protect the virus from immune recognition. However, little

evidence has been presented in actual support of this hypothesis.

It is to be understood that terms used throughout this specification shall have their

ordinary meaning unless defined herein.

"Viral infectivity", as used herein, refers to the ability of an infective virus containing an

15   envelope gene of HIV, or an infectious DNA clone, that is engineered to encode the mutated

consensus amino acid recognition sequence for N-linked carbohydrate attachment, to replicate

in culture or in vivo.

Wild-type or native HIV-1 envelope glycoprotein refers to the envelope glycoprotein

encoded by a naturally occurring HIV-1 isolate. With respect to designation of amino acid

20   positions of the envelope glycoprotein such as the Cys at the N-terminal side of the cysteine loop

containing V3 (approximately amino acid position 296) or the Cys at the C-terminal side of the

cysteine loop containing V1 and V2, it is understood that certain aspects of envelope structure

are conserved throughout virtually all HIV-1 strains, and those conserved structures can be used

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as landmarks. For example cysteine cross-links form loops which contain hypervariable regions in gp120 having widely accepted designations.

"Recombinant glycoprotein" refers to a glycoprotein produced by expression of a DNA sequence that does not occur in nature and which results from human manipulations of DNA bases. The term recombinant envelope glycoprotein means gp160, gp120, or other env-encoded peptides containing at least the above-described N-terminal portion of gp120 and containing at least one and if desired multiple mutated N-linked carbohydrate attachment sites as described herein.

As used herein, a recombinant protein or epitope of a protein is "immunogenic" or "antigenic" when it elicits an antibody response or is recognized by immunocompetent cells (i.e., cells of the immune system). An antibody response is indicated by the formation in a mammal of antibodies to the protein and can be detected by conventional antibody detection assays on serum from the mammal; e.g., an ELISA. Recognition of immunocompetent cells is indicated when the protein or epitope triggers activation of such cells, as measured in terms of proliferation and/or induction of effector functions, e.g., as measured by production of lymphokines, cytokines, and/or killing of cells expressing the protein or epitope. Therefore, a protein or epitope is "non-immunogenic" (non-antigenic) when it is not able to elicit an antibody response or does not trigger the activation of immunocompetent cells, as explained above. A recombinant protein of the invention may be determined to be infective and therefore useful in further testing of infectivity in animals if it promotes syncytium formation as described herein.

Recombinant envelope glycoproteins according to the invention are recombinant human immunodeficiency virus envelope glycoproteins which are mutated with respect to a wild type (native) human immunodeficiency virus glycoprotein in the primary amino acid sequence to effect partial underglycosylation of the molecule. The term "envelope glycoproteins" include the full

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length proteins or fragments thereof retaining the activity of the full length envelope glycoprotein. It is to be understood, however, that the term "underglycosylation" also refers to nonrecombinant HIV envelope glycoproteins which may undergo removal of glycans through standard known techniques to produce underglycosylated HIV envelope glycoproteins, rather than through recombinant techniques. Proteins according to the invention will contain an amino acid sequence alteration which is introduced to positions in the N-terminal portion of gpl20 or useful fragments thereof (between the N-terminus of gpl20 and a specific cysteine at the N-terminal side of V3 which forms the loop containing V3). When recombinant envelope glycoproteins according to the invention are present as a component of the virion, the virion is infective. Furthermore, in individuals immunized with this recombinant envelope glycoprotein molecule, a fragment thereof containing the mutated amino acid sequence, or a virion containing the mutated amino acid consensus sequence, or DNA encoding the recombinant envelope glycoprotein or mutated portion thereof, an immune response will be induced to reduce or block viral infectivity.

As illustrated by the studies described below, potential N-linked glycosylation sites in gp120 itself or as a component of gp160, gp140 or other useful fragments thereof can be systematically mutated, either singly or in combination by site directed mutagenesis such that the consensus glycosylation sequence is disrupted. Recombinant viruses are generated containing gpl20 genes that have such mutations. To determine whether the conformation is retained in the mutated gpl20, the infectivity of each mutant virus is measured. Processing of gpl60 to gpl20 and gp41 may also be assessed as a rough measure of retention of conformation and infectivity.

In general there are more than 20 consensus N-linked glycosylation sites in the gp120 coding sequence of HIV-1 isolates. The relative positions of these sites on gpl20 in HXB2 and in other strains of HIV-1 are illustrated in Fig. 1. A linear map of the conserved N-linked glycosylation sites, their relative positions and their level of conservation are presented in Fig.2.

In Fig. 2, the following residue designations correspond to the arrows of gp120:

a1 = 88	a5 = 241	a9 = 356
a2 = 136	a6 = 262	b9 = 386
b1 = 141	a7 = 276	b11 = 392
a3 = 156	b5 = 289	b12 = 397
b2 = 160	a8 = 296	b13 = 406
a4 = 186	b6 = 301	a10 = 448
b3 = 197	b7 = 332	a11 = 463

230 not marked

b4 = 234   b8 = 339

While the sites listed above may be singly mutated, it is to be considered an additional and advantageous aspect of the invention that at least one or more of the sites in HIV-1gp120 be mutated.

Sequence information for envelope proteins of other strains (e.g. the strains listed above) are referenced in Myers et al. Human Retroviruses and AIDS (1991): "A compilation and analysis for nucleic acid and amino acid sequences" (Los Alamos National Laboratory, Los Alamos, NM), which is hereby incorporated by reference in its entirety.

Because underglycosylation may unmask envelope regions and make them immunogenetic, it is possible to use any of a wide range of HIV-1 strains or isolates in the practice of the present invention, e.g., MN, HXB2, LAI, NL43, MFA, BRVA, SC, JH3, ALAI, BALI, JRCSF, OYI, SF2, NY5CG, SF162, JFL, CDC4, SF33, AN, ADA, WMJ2, RF, ELI, Z2Z6, NDK, JYI, MAL, U455, Z321. The preferred mutation at the consensus N-linked glycosylation sequence is substitution of Asn, Ser, or Thr with a different amino acid defined as any amino acid other than the one occupying the position in the wild type.

Preferably, there are multiple underglycosylations in the above described N-terminal region, particularly in the region between the N terminus of gp120 and the Cys on the N-terminal side of the cysteine loop containing hypervariable region 3 (V3).

It is preferred according to the invention that sites of N-linked carbohydrate attachment located in the C-terminus of the gp120 molecule (i.e., from about amino acid 296 to the C-terminus of the glycoprotein) are not mutated as described herein; that is, the majority of such sites in the C-terminal half of the molecule retain their function with respect to carbohydrate attachment, leaving the molecule substantially glycosylated in its carboxy terminal half. However, it is within the scope of the invention to provide a recombinant HIV-1 envelope glycoprotein which contains a mutated N-linked carbohydrate attachment site in the N-terminal half of the molecule and which also contains one or several mutated N-linked carbohydrate attachment sites in the C-terminal half of the molecule. For example, a recombinant HIV-1 envelope glycoprotein according to the invention may contain a mutated N-linked carbohydrate attachment site within the N-terminal half of the molecule in combination with a mutated N-linked carbohydrate attachment site at one or more of the positions located in the C-terminal half of the envelope glycoprotein; such as one or more of sites 386, 392, 397, 406 or 463, and also optionally including mutated consensus sequences at approximately position 448 and/or position 392. For convenience the numbers given above for gp120 refer to amino acid residues of the HXB2 envelope protein.

Those skilled in the field will understand that conservation of envelope features in other strains will permit the application of the invention to the envelope proteins of those strains. For example, there is conservation of cysteine cross-links that define loops with hypervariable regions. Thus, the reference to positions 386, 392, 397, 406 and 463 can be understood as a reference to the N-linked glycosylation sites positioned between the C-terminus of gp120 and the Cys on the

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N-terminal side of the cysteine loop containing hypervariable region 4 (V4). Similarly, the reference to positions 289 and 356 can be applied to other strains with reference to Fig. 1 and Fig. 2.

The invention also provides mutated sites of N-linked carbohydrate attachment in an HIV-1 envelope glycoprotein such as gp160, truncated forms of gp 160 such as gp 140, or gp120, or fragments thereof which altered glycoproteins are effective HIV-1 vaccines. These useful molecules according to the invention are prepared as follows.

N-linked glycosylation sites can be identified by locating the amino acid consensus sequence Asn-X-Ser/Thr in the glycoprotein. The corresponding nucleotide sequence is located in the DNA sequence encoding the glycoprotein. The corresponding nucleotide sequence to the amino acid consensus sequence is then mutated such that the codon specifying any one or more of the amino acids of the consensus sequence is altered so as to specify an amino acid other than the consensus amino acid. The altered DNA sequence can then be used to produce an altered envelope glycoprotein or can be assembled into the DNA of the HIV-1 virion, along with the altered envelope protein, or into a vaccinia virus as known in the art and described herein. Recombinant virions containing the altered glycoprotein and altered nucleotide sequence, wherein the mutations have substantially no effect on infectivity, can then be identified according to methods and procedures well known in the art.

More specifically, the molecular clone HXB2, which contains 24 N-linked glycosylation sites is used as the template DNA for site-directed mutagenesis as follows. Oligonucleotide-directed mutagenesis is performed on a selected fragment of HXB2 (Cohen et al., 1990 *J. AIDS* 13:11), which covers all 24 N-linked glycosylation sites of gp120, using the method of Kunkel (Cohen et al., 1988, *Nature* 334:532). The oligonucleotide primers used for mutagenesis are synthesized using standard cyanoethyl phosphoamadite chemistry and are listed in Table I below.

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Mutants are identified by the Sanger chain-termination method (Cullen, 1986, *Cell* 46:973). The

fragment containing the desired mutation is excised from the replicative form of each mutant and

used to replace the same fragment of HXB2. All HXB2-derived N-linked glycosylation site

mutants containing the designated changes are further verified by DNA sequencing (Cullen, 1986,

5   *Cell* 46:973).

TABLE 1

Mutant Infectivity Virus	Amino Acid Change	Mutagenic Oligonucleotide (5' to 3')
88	Asn to Gln	
TAGTATTGGTA<u>CA</u>G<u>G</u>TGACAGAAAATTT		
136	Asn to Gln	TGATTTGAAGC<u>A</u>G<u>G</u>ATACTAATAC
141	Asn to Gln	
ATACTAATACC<u>CA</u>A<u>A</u>AGTAGTAGCGGGA		
156	Asn to Gln	GATAAA<u>C</u>A<u>G</u>TGCTCTTTCAATAT
160	Asn to Gln	CTGCTCTTTC<u>C</u>A<u>G</u>ATCAGCACAAG
186	Asn to Gln	TACCAATAGATC<u>A</u>G<u>G</u>ATACTACCAGC
197	Asn to Gln	TGACAAGTTGTC<u>A</u>G<u>A</u>CCTCAGTCAT
230	Asn to His	TAAAATGTAAT<u>C</u>ATAAGACGTTCA
234	Asn to His	ATAAGACGTTC<u>C</u>ATGGAACAGGACCA
241	Asn to Gln	
GACCATGTACA<u>C</u>A<u>G</u>GTCAGCACAGTAC		
262	Asn to Gln	ACTGCTGTTA<u>C</u>AAGGCAGTCTAG
276	Asn to Gln	TTAGATCTGTC<u>C</u>A<u>G</u>TTCACGGACAAT
289	Asn to Gln	
TAGTACAGCTG<u>C</u>A<u>G</u>ACATCTGTAGAAA		
295	Asn to Gln	CTGTAGAAATTC<u>A</u>A<u>T</u>GTACAAGAC
301	Asn to His	ACAAGACCCAAC<u>C</u>ACAATACAAGAAA
332	Asn to His	GCACATTGT<u>C</u>ACATTAGTAGAGC
339	Asn to Gln	GCAAATGG<u>C</u>A<u>G</u>AACACTTTAAAAC
356	Asn to Gln	
TTCGGAAAT<u>C</u>A<u>G</u>AAAACAATAATCTTTA		
386	Asn to Gln	TTTCTACTGT<u>C</u>A<u>G</u>TCAACACAACTG
392	A s n t o G l n	
ACAACTGTTT<u>C</u>A<u>G</u>AGTACTTGGTTTAATAG		
397	Asn to Gln	GTACTTGGTTT<u>C</u>A<u>G</u>AGTACTTGGAG
406	Asn to Gln	CTGAAGGGTCA<u>C</u>ATAACACTGAAGGA
448	Asn to Gln	GATGTTCATCA<u>C</u>A<u>G</u>ATTACAGGGCTG
463	Asn to His	GGTAATAGCAAC<u>C</u>ATGAGTCCGAGAT

Recombinant HIV-1 envelope glycoprotein molecules according to the invention which are candidate vaccine molecules will possess the following properties: 1) they will be altered in their primary amino acid sequence at one or more selected sites in the N-terminal portion of the molecule such that the site is no longer recognized in a mammalian, and preferably a human cell,

5   as a site of carbohydrate attachment; 2) the sequence alterations to the protein will alter the protein to an extent which permits immune recognition of the protein; and 3) a sufficient amount of the wild type conformation of the molecule should be retained such that the mutant virus substantially retains infectivity. It is believed that a recombinant gp120 molecule which satisfies these criteria will be more likely to elicit a protective immune response against wild-type HIV-1

10   strains and thus to reduce infectivity of the natural virus.

Recombinant gp120 molecules derived from any strain of HIV-1 which satisfy the criteria listed above can be generated using the methods described above. In order to carry out the invention as to any strain of HIV-1, one of skill in the art needs to know the sequence of the gp120/gp160 gene in the particular strain of HIV-1 of interest. The sequences of gp120/gp160 of

15   many strains of HIV-1 are known; where new strains are discovered, the gp120/gp160 sequence may be determined by a skilled artisan using ordinary cloning and sequencing technology such as that described in the Molecular Cloning Manual (Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory*, NY). Potential vaccine molecules can be obtained by the skilled artisan without undue experimentation because the techniques and tests

20   to be used are common and familiar to those knowledgeable in the art and are described herein to the extent that they are needed to practice the invention.

Described herein are materials and methods for generating gp120 molecules containing an altered amino acid sequence according to the invention and then determining their ability to act as vaccines. It is to be understood that altered gp160 molecules or fragments thereof also are

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useful according to the invention as a vaccine candidate provided the N-terminal end of the gp120

portion of the gp160 molecule is underglycosylated. Altered gp160 molecules can be generated

using the procedures described herein for gp120.

5   **Determination of Effect of Sequence Alteration on Carbohydrate Addition**

The invention contemplates alteration of the primary amino acid sequence of an HIV-1

envelope glycoprotein such that at least one site in the N-terminus of the envelope glycoprotein

is no longer recognized as an N-linked carbohydrate addition site and therefore not glycosylated

when the protein is synthesized in a mammalian, and preferably a human cell. In order to

10   determine whether the recombinant protein molecule is sufficiently underglycosylated to become

immunogenic, the mobility of the recombinant protein on a gel is compared to the mobility of the

wild type protein. Where the gel mobility of the recombinant protein differs from the wild type

protein by a visible shift in band migration, it can be assumed that the recombinant protein is

underglycosylated to an extent which is sufficient to test the recombinant molecule further for

15   immunogenicity. Alternatively, chemical techniques for quantitating sugar content are well

known. See, e.g., Chapin at al. IRL Press (1986) pp. 178-181 and *Methods of Carbohydrate*

Chemistry Vol. 7 (Whistler at al. Eds.) Academic Press (1976) p. 198 which describe acid

hydrolysis and methanolysis. After methanolic hydrolysis, monosaccharides are derivatized e.g.,

to trimethysilyl ethers of the methyl glycosides. Quantitation is accomplished by gas

20   chromatography using parallel external standards of monosaccharide mixtures. Alternatively total

sugar content of a glycoprotein of known amino acid sequence can be determined by mass

spectroscopy to obtain accurate mass of glycosylated and unglycosylated moieties.

Expression of Recombinant HIV-1 Envelope Glycoprotein

Recombinant gp120 or gp160 mutant glycoproteins can be obtained by expressing these proteins in any one of a number of expression systems. These systems include but are not limited

5   to the following.

A baculovirus expression system can be used to obtain recombinant gp120 or gp160. A gene encoding the recombinant glycoprotein can be cloned into a commercially available baculovirus transfer plasmid. A recombinant baculovirus encoding such a protein can be generated as described by Summers and Smith (1988, *A Manual of Methods for Baculovirus*

10   *Vectors and Insect Cell Culture Procedures: Texas Agricultural Experiment Station Bulletin No. 1555*, College Station, Texas). The virus can be used to infect cells, such as Sf9 cells, whereupon the recombinant glycoprotein will be expressed to high levels as the baculovirus replicates. Protein is recovered from the culture using ordinary standard biochemical techniques.

Alternatively, Chinese hamster ovary (CHO) cells can be transfected with a plasmid

15   encoding a mutated gp120 or gp160 gene, using any number of transfection methods all of which are described in detail in Sambrook at al. (supra). Recombinant proteins can be expressed in a constitutive manner under the control of its own promoter or under the control of another promoter such as another retrovirus LTR. Alternatively, recombinant proteins can be expressed in an inducible manner, wherein expression is driven by a promoter that responds to the addition

20   of an inducer molecule to the transfected cells. Examples of such promoters can be found in Sambrook at al. (Supra). Glycoproteins that are so expressed can be recovered from the cells and from the cell medium using common biochemical techniques. See Lasky et al. *Science* 233:209-212 (1986); Robey et al. *Proc. Nat'l. Acad. Sci.* 83:7023-7027 (1986); Pyle et al. *Aids Research and Human Retrovirus* 3:387-399 (1987).

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Proteins of the invention can also be produced as part of a viral particle, with or without alterations to other portions of the virus. See, e.g., the method of Aldovini et al. *J. Virol.* 64:1920-1926 (1990).

5   <u>Monitoring of syncytium-formation and viral infectivity</u>

To evaluate whether mutations introduced into any of the individual N-linked glycosylation sites affected syncytium-formation and viral infectivity, cell-free virions obtained from the culture supernatant of COS-1 transfectants are collected at 48 hours post-transfection. Equal amounts of mutant and wild type viruses, as measured by RT activity, are used to infect

10   CD4-positive SupT1 cells. Virus-infected cultures are monitored for syncytium formation as determined by the presence of multinucleated cells as a measure of viral infectivity. As in the case of the wild type virus-infected SupT1 cultures, syncytia and RT activity are expected to be detected in all the mutant virus-infected SupT1 cultures.

The CD4 positive human T lymphoid cell line, SupT1, is grown and maintained at 37°C

15   in RPMI-1640 containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. COS-1 cells are propagated in Dulbecco's minimal eagle medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. Cell-free supernatants are collected 48 hours after transfection. Supernatants are filtered through 0.45 mm filters and assayed for virion-associated reverse transcriptase (RT) activity. Equal amounts of

20   wild type and mutant virus, as measured by RT activity (100K cpm), is used to infect 1×10^6 SupT1 cells. One milliliter of the culture medium is collected every three or four days and assayed for RT. Cultures are monitored for 28 days to determine syncytium formation as a measure of viral infectivity.

Reverse transcriptase assay to determine growth kinetics

One milliliter of culture medium is mixed with 0.5ml 30% PEG and 0.4M NaCl on ice for

2 hours and spun at 2500 rpm at 4° C for 30 minutes. The pellet is resuspended in 100 ml of RT

buffer (0.5% Triton X-100, 15mM Tris pH 7.4, 3mM dithiothreitol, 500mM KCL, 30% glycerol).

5   Ten micro liters of the solution is incubated with 90Ml of RT cocktail (40 mM Tris HCL, pH 7.8,

10mM $MgCl_2$, 8mM dithiothreitol, 94ml ddH_2O, 0.4 U Poly (rA) oligo (dT) [optical density at

260nm] per ml and 2.5 mCi/ml ^{3}H-labeled dTTp) at 37° C for 1.5 hours. The reaction mixture

is precipitated with 3ml of 10% trichloroacetic acid (TCA) and 10ml of 1% tRNA which served

as the carrier, and is then chilled on ice for 20 minutes. The reaction mixture is filtered through

10   Whatman GF/C glass microfiber filters and washed 3 times with 5% TCA to remove

unincorporated ^{3}H-dTTP. Radioactivity is measured in a liquid scintillation counter.

Single Mutants in gp120

The ability of HXB2-derived mutants (each having one of the 24 N-linked glycosylation

15   sites mutated by site-directed mutagenesis) to infect CD4-positive SupT1 cells is compared with

that of the wild type virus. Most of the individual consensus N-linked glycosylation sites are

dispensable for viral infectivity. N-linked glycosylation sites that are likely to play important roles

in HIV-1 infectivity are not randomly distributed in gp120; they are generally located in the N-

terminal half of gp120.

20   Since underglycosylation of proteins can improve their immunogenicity, a candidate

vaccine for HIV-1 might be a partially glycosylated gp120 with most of the dispensable N-linked

glycosylation sites removed, such that the conformation of the protein is largely unaltered and the

CD4 binding site is retained.

Each of the 24 potential N-linked glycosylation sites in the gp120 coding region of the

infectious molecular clone HXB2, is individually modified to generate 24 N-linked glycosylation

site mutants (See Table 1). In these mutants, the Asn-X-Ser/Thr attachment sequence is replaced

by either Gln-X-Ser/Thr or His-X-Ser/Thr. The underlying hypothesis is that if a given N-linked

5   glycosylation site played no significant role in syncytium-formation or viral infectivity, then such

a mutant should retain its infectivity and its ability to form syncytia. Each of the 24 mutants is

designated by the residue number of the respective N-linked glycosylation site as presented in

Table 1.

10   ### Expression of envelope proteins

To determine if mutations introduced to any of the 24 N-linked glycosylation sites affected

the expression of the envelope protein, 10 μg each of mutant or wild type proviral DNA is

transfected in 3-5 x 10^6 COS-1 cells using DEAE-dextran as described above. Cell lysates derived

from COS-1 transfectants are then examined in standard western blots. It is expected from this

15   example that no particular individual N-linked glycosylation site is indispensable for the expression

of the envelope protein.

Generation of Antibodies

20   Recombinant envelope proteins can be used to generate antibodies using standard

techniques, well known to those in the field. For example, the proteins are administered to

challenge a mammal such as a monkey, goat, rabbit or mouse. The resulting antibodies can be

collected as polyclonal sera, or antibody-producing cells from the challenged animal can be

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immortalized   (e.g. by fusion with an immortalizing fusion partner) to produce monoclonal

antibodies.

Preparation of Antibodies

5   1. Polyclonal antibodies.

The recombinant protein may be conjugated to a conventional carrier in order to increase

its immunogenicity, and antisera to the peptide-carrier conjugate is raised. Coupling of a peptide

to a carrier protein and immunizations may be performed as described in Dymecki, S.M., et al.,

J. Biol. Chem. 267:4815-4823, 1992. The serum is titered against protein antigen by ELISA or

10   alternatively by dot or spot blotting (Boersma and Van Leeuwen, 1994, *J. Neurosci. Methods*

51:317).   At the same time, the antisera may be used in tissue sections. The sera is shown to

react strongly with the appropriate peptides by ELISA following the procedures of Green et al.,

Cell, 28, 477-487 (1982).

2. Monoclonal antibodies.

15   Techniques for preparing monoclonal antibodies are well known, and monoclonal

antibodies of this invention may be prepared using a recombinant envelope glycoprotein described

herein or a synthetic peptide thereof containing the altered amino acid sequence, preferably bound

to a carrier, as described by Arnheiter et al., *Nature*, 294, 278-280 (1981).

Monoclonal antibodies are typically obtained from hybridoma tissue cultures or from

20   ascites fluid obtained from animals into which the hybridoma tissue was introduced. Nevertheless,

monoclonal antibodies may be described as being "raised to" or "induced by" the synthetic

peptides or their conjugates.

Particularly preferred immunological tests rely on the use of either monoclonal or

polyclonal antibodies and include enzyme linked immunoassays (ELISA), immunoblotting,

immunoprecipitation and radioimmunoassays. See Voller, A., *Diagnostic Horizons* 2:1-7, 1978,

Microbiological Associates Quarterly Publication, Walkersville, MD; Voller, A. et al., *J. Clin.*

Pathol. 31:507-520 (1978); U.S. Reissue Pat. No. 31,006; UK Patent 2,019,408; Butler, J.E.,

Meth. Enzymol. 73:482-523 (1981); Maggio, E. (ed.), *Enzyme Immunoassay*, CRC Press, Boca

5   Raton, FL, 1980) or radioimmunoassays (RIA) (Weintraub, B., *Principles of*

Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine

Society, March 1986, pp. 1-5, 46-49 and 68-78). For analyzing tissues for the presence of the

recombinant protein of the present invention, immunohistochemistry techniques are preferably

used. It will be apparent to one skilled in the art that the antibody molecule will have to labeled

10   to facilitate easy detection of mutant protein. Techniques for labeling antibody molecules are well

known to those skilled in the art (see Harlour and Lane, *Antibodies*, Cold Spring Harbour

Laboratory, pp 1-726, 1989).

Alternatively, other techniques can be used to detect the mutant proteins, including

chromatographic methods such as SDS PAGE, isoelectric focusing, Western blotting, HPLC and

15   capillary electrophoresis.

Monoclonal antibody-producing hybridomas (or polyclonal sera) can be screened for

antibody binding to the protein and to wild type envelope. They can also be screened for the

ability to neutralize infectivity of HIV-1 isolates, preferably multiple (e.g., at least 3) isolates each

having diverse sequences in the hypervariable V1 or V2 regions.

20   By antibodies we include constructions using the binding (variable) region of such

antibodies, and other antibody modifications. Thus, an antibody useful in the invention may

comprise whole antibodies, antibody fragments, polyfunctional antibody aggregates, or in general

any substance comprising one or more specific binding sites from an antibody. The antibody

fragments may be fragments such as Fv, Fab and F(ab')$_2$ fragments or any derivatives thereof, such

as a single chain Fv fragments. The antibodies or antibody fragments may be non-recombinant, recombinant or humanized. The antibody may be of any immunoglobulin isotype, e.g., IgG, IgM, and so forth. In addition, aggregates, polymers, derivatives and conjugates of immunoglobulins or their fragments can be used where appropriate.

5   The following examples are set forth as representative of the present invention. These examples are not to be construed as limiting the scope of the invention as these and other equivalent embodiments will be apparent in view of the present disclosure, figures, tables, and accompanying claims.

10   **EXAMPLE I**

Described below are experiments which demonstrate the extent to which N-linked glycosylation sites in the V1 region of gp120 of SIVmac239 are dispensable for viral replication.

Site-specific mutagenesis and subcloning were performed as follows. In order to reduce the size of the plasmids to be mutated, the Sph-Cla 1 fragment of the proviral SIVmac239 DNA containing 1469 nucleotides of env coding sequence (proviral nucleotide numbers 6450-8073 in
15   Regier and Desrosiers, *AIDs Research and Human Retroviruses*, 6:1221-1231, 1990) was subcloned into pSP72 (Promega) resulting in pSP72SC. The Sac 1-EcoR1 fragment containing the 3' 1050 bases of the proviral genome was subcloned into pSP72 to create pSP72SE. Mutations of env were created by recombinant PCR mutagenesis (Du, BioTechniques). The
20   mutagenic primers used were:

(6931- 6969) 5'-ACTATGAGATGCCAGAAAAGTGAGACAGATAGATGGGGAT-3' and (6957-6919)
5'-TGTCTCACTTTTCTGGCATCTCATAGTAATGCATAATGG-3' for g4;
(7027-7068)5'-GTAGACATGGTCCAGGAGACTAGTTCTTGTATAGCCCAGGAT-3' and (7053-7014)
5'-AGAACTAGTCTCCTGGACCATGTCTACTTTTGCTGATGCT-3' for g5; (7057-7097)

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5'-ATAGCCCAGGATCAATGCACAGGCTTGGAACAAGAGCAAAT-3',   (7084-7045)   5'-

CCAAGCCTGTGCATTGATCCTGGGCTATACAAGAACTAGT-3' for g6;

(7026-7062)   5'-AGTAGACGTGGTCAATGAGACTAGTTCTTGTATAGCC-3'   and   (7045-7010)

5'-GTCTCATTGACCACGTCTACTTTTGCTGATGCTGTCG for M144V;

5   (7022-7055)   5'-CAAAAGTAGACGTGGTCCAGGAGACTAGTTCTTG   and   (7042-7009)

5'-CCTGGACCACGTCTACTTTTGCTGATGCTGTCGT for g456(M144V).

Primers were synthesized on a Cyclone DNA synthesizer (Biosearch, Inc.) or were

purchased from Genosys Biotechnologies, Inc. (Woodlands, Texas). Proviral nucleotide sequence

numbers are according to Regier and Desrosiers (ibid). The Sph1-Cla1 fragment containing the

10   mutated env sequence was excised and subcloned into the 3' parental clone, pSP72-239-3'

(Ilyinskii and Desrosiers 5/96). For transient gene expression, the wild-type envelope sequence

was subcloned into the Xho1 and BamH1 sites of the expression vector pSVL (Pharmacia)

following creation of a BamH1 site 3' of the env coding sequence using the mutagenic primers

#27 (9268-9302) 5'- GTATATGAAGGATCCATGGAGAAACCCAGCTGAAG-3' and #28

15   (9286-9253) 5'- CCATGGATCCTTCATATACTGTCCCTGATTGTAT-3'.   The mutant

envelope sequences were subcloned into the resultant pSVLenv via the unique Xho1 and Sac1

sites.

EXAMPLE II

20   DNA transfection of cultured cells was performed as follows. The 5' and 3' clones of

SIVmac239 were digested with Sph1 and heated to 65°C for 15 minutes. Each right-half clone

was ligated together with the left- half clone p239SpSp5' using T4 DNA ligase.   Three

micrograms of the ligated DNA was used to transfect CEMx174 cells treated with DEAE-dextran

(Naidu, 1988). For transient expression in COS-1 cells, the pSVL vector containing the wild-type

25   or mutant envelope sequences were transfected into DEAE-dextran treated COS-1 cells, 1

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microgram of DNA was used following the procedure of Levesque et al. (Levesque, J.-P., P.

Sanilvestri, A. Hatzfeld, and J. Hatzfeld (1991) DNA transfection in COS cells. *BioTechniques*

11:313-318.)

5   **EXAMPLE III**

Virus stocks and cell culture were prepared as follows. Rhesus monkey peripheral blood

mononuclear cells (PBMC's), CEMx174, 221, and COS-1 cells were maintained as previously

described. For virus stocks, CEMx174 cells were transfected as described above. The medium

was changed every 2 days and the supernatants were harvested at or near the peak of virus

10   production. Cells and debris were removed by centrifugation and virus contained in the

supernatant was aliquoted and stored at - 70°C. The concentration of p27 antigen was measured

by antigen capture assay (Coulter Corporation, Hialeah, FL). For virus infections, five

micrograms of p27 was used to infect 2.5 million pelletted cells.

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                                               EXAMPLE IV

DNA sequencing and PCR amplification was performed as follows. Cloned fragments

containing mutated DNA were sequenced in their entirety manually or with an ABI377 automated

20   DNA sequencer using dye-terminator cycle sequencing chemistry according to the instructions

of the manufacturer (Perkin-Elmer Inc., Foster City, Calif.). Total genomic DNA was isolated

with the HRI AmpPrep kit (HRI Research, Inc., Concord, Calif) and used as a template for nested

PCR amplification, using primers located outside of the viral env sequence. Outer primers were

#39   5'-GAGGGAGCAGGAGAACTCATTAGAATCCTCC-3'   and   #40   5'-

GTTCTTAGGGGAACTTTTGGCCTCACTGATACC-3'. The inner mutagenic primers created

Xho1 and BamH1 sites used for cloning the PCR products into pSP72 and were #38

5'-CTCAGCTATACCTCCCTCGAGAAGCATGCTATAAC-3'   and   #32   5'-

CTCCATGGATCCTTCATATACTGTCCCTGATTG-3'. Each 100 μl reaction mix contained

one microgram total DNA, 2mM Mg^{+2}, 200 μM each of the four deoxynucleoside triphosphates,

0.2 μM each primer, and 2 U of Vent polymerase (New England Biolabs, Beverly, Mass.) and

were amplified for 30 cycles. Each cycle consisted of denaturation at 93°C for 1 min, annealing

at 50°C for 1 min, and elongation at 72°C for 3 min 15 s ending with a 10 min final extension at

72°C for the last cycle.

EXAMPLE V

Immunoblotting and CD4 binding were carried out as follows. For western blot analysis,

COS-1 cells at 80-90% confluence in 35 mm diameter plates (Falcon Primaria) were rinsed three

times with PBS and lysed in 0.5ml lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 10

mM NaCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM PMSF, 1 mM

Pefabloc (Boehringer) and 1 mg iodoacetamide). Following electrophoresis, the proteins were

transferred onto a PVDF membrane (Millipore Corp.) and treated sequentially with a rhesus

polyclonal antibody generated against SIVmac239 followed with a horseradish

peroxidase-conjugated anti-rhesus IgG (Southern Biotechnology Associates, Alabama). The

proteins were subjected to a chemiluminescent substrate (ECL Reagents, Amersham Int'l.,

England) and immediately detected by being placed against film (Kodak BioMax) for 5-200 s.

For metabolic labeling, monolayers were washed once with labeling medium (minimum essential

medium without methionine and cysteine plus 10% dialyzed fetal calf serum), and then incubated

with 1 ml of same medium containing 100 uCi of 35S labeled methionine and cysteine (NEN,

Boston, Mass.) for 16 hours. The cells were washed twice in PBS and lysed in 0.5 ml lysis buffer.

All lysates were frozen at -20°C, thawed, vortexed vigorously, and the cell debris was pelleted

by centrifugation for 2 minutes. For CD4 binding assays, 100 ul of each lysate was incubated with

either PBS or 250 ng of soluble CD4 as described previously (Morrison et al., Virl. 1995).

5   **EXAMPLE VI**

Experimental infection of rhesus monkeys was carried out as follows. CEMx174-derived

virus stocks containing 50 ng of p27 were used for intravenous inoculation of juvenile rhesus

monkeys (Macaca mulatta). Two animals were infected with each of the SIVmac mutants and

with SIVmac239.

10   ELISA assays were performed as previously described (*Techniques in HIV Research*,

Eds.: A. Aldovini and B. Walker. Stockton Press, 1990, NY, pp.121-127) Peptide 1 was

purchased from Bio-Synthesis,Inc., (Lewisville, TX) and consisted of the amino acid sequence

NH2- Cys Asn Lys Ser Gle Thr Asp Arg Trp Gly Leu -COOH.

As shown by schematic in Fig. 3, the 4th, 5th, and 6th glycosylation sites containing the

15   consensus sequence Asn X Ser/Thr in the gp120 sequence of SIVmac239 were selected for

mutagenesis. These sites are located in the N-terminal half of the gp120 molecule and in the

vicinity of the highly variable region 1 but nonetheless are strongly conserved among SIV

sequences. Therefore, the 4th, 5th and 6th sites are representative sites for mutation and testing

of the resultant altered gp120 or gp160 molecule according to the invention.

20   The Asn codon at all three sites of SIVmac239 is AAT. The AAT at sites 4 and 5 were

changed to CAG (Gln) and at site 6 it was changed to CAA (Gln). Gln is structurally similar to

Asn, differing only by a single CH_2 group. Since only AAT and AAC can code for Asn, two

nucleotides would be required in the codon to revert back to Asn. All seven possible mutant

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forms of these sites were created. These will be referred to as g4, g5, g6, g45, g46, g56, and g456 as indicated in Fig. 3.

All six single and double mutants (g4, g5, g6, g45, g46, g56) replicated similar to the parental virus upon transfection of cloned DNA into CEMx174 cells (data not shown).

5   Normalized amounts of mutant and parental virus stocks produced from CEMx174 transfection were used to analyze viral replication in CEMx174 cells, the rhesus monkey 221 cell line, and in primary rhesus monkey PBMC cultures. As shown by the data presented in Figs. 4 and 5, all single and double mutant forms of virus replicated similar to the parental virus in CEMx174 cells, 221 cells (data not shown) and stimulated rhesus monkey PBMC cultures as shown by the data

10   in Fig. 6. Slight delays or differences in peak heights were observed with the mutants in some experiments but it is uncertain whether these represent a significant difference.

In contrast to the results presented above using the single and double mutants, replication of the triple mutant (g456) was severely impaired. In one CEMx174 culture transfected with g456 DNA, detectable virus began to appear beyond 40 days after transfection as shown by the

15   data in Fig. 7. When virus derived from day 57 of this transfection was used to infect CEMx174 cells, virus replicated with only a slight delay when compared to parental virus as shown by the data in Fig. 8.   These findings suggested that revertants had appeared in the culture to allow wild-type or near wild-type levels of viral replication.

As shown in Fig. 9, sequence analysis of viral DNA derived from CEMx174 cells infected

20   with the g456 revertant revealed a single predominant change of Met to Val at position 144. This position is located two amino acids upstream of the mutated 5th N-linked site. No changes were observed in the 4th, 5th and 6th QXS/T sites themselves as shown in Fig. 9. We introduced the Val to Met change into the parental SIV239 DNA and into the g456 mutant in the absence of any other changes. Virus containing the M144V change in the 239 background replicated similar to

the parental SIVmac239 upon both transfection and infection in both CEMx174 and 221 cells as shown by the data in Figs. 10 and 11. As also shown in Figs. 10 and 11, virus containing the M144V change in the g456 background replicated with only a slight delay when compared to SIVmac239 upon both transfection and infection in both CEMx174 and 221 cells. The M144V

5   mutant in the g456 background replicated with similar kinetics to the revertant recovered from the original transfection shown in Fig. 7. Thus, the change of Met to Val at position 144 is able to compensate for the loss of the 4th 5th and 6th NXS/T sites.

Vectors for transient expression of SIV 239 and g456 envelope proteins were constructed and transfected into COS-1 cells. Envelope protein was detected by Western blot. As shown in

10   Fig. 12, the SIV 239 clone yielded both the gp160 precursor and gp120 external surface subunit as expected during the 2-5 day time period that was examined. The g456 mutant yielded a precursor that migrated slightly faster than the gp160 precursor of the wild type. Such a faster mobility would be expected for a protein lacking some of its N- linked glycosylation. Little or no processed forms of env protein with a slightly faster mobility than gp120 were detected during

15   the 2-5 days of measurement.

The three mutant strains of SIV constructed in which 1) the 4th and 5th glycosylation sites were altered; 2) the 4th and 6th glycosylation sites were altered; and 3) the 5th and 6th glycosylation sites were altered, retained their ability to replicate and, hence, demonstrated viral infectivity. Two monkeys were infected with each of the three mutant strains and two monkeys

20   were infected with parental wild type SIV. All eight monkeys mounted antibody responses that were similar in titer to wild virus. However, the two monkeys that were infected with the mutant missing the 4th and 5th glycosylation sites and the two monkeys that were infected with the mutant missing the 4th and 6th glycosylation sites had significantly stronger antibody responses to an 11 amino acid stretch that included the 4th site. This significant and unexpected result is

shown in Fig. 14 and demonstrates that a virus with a gp120 containing an altered carbohydrate attachment site so as to prevent glycosylation at that site in the N-terminal half of the gp120 molecule is very immunogenic, whereas a wild type virion is much less immunogenic. These results indicate that HIV-1 molecules which contain such N-terminal N-linked carbohydrate addition site alterations in the HIV-1 envelope glycoprotein are good candidates for HIV-1 vaccines.

Another SIV strain has been identified that is missing each of the 5th, 6th, 8th, 12th, and 13th sites of carbohydrate attachment. This mutant virus is replication competent as show in Fig. 13. Studies have confirmed that mutants underglycosylated individually at the 4-13 glycosylation sites are replication competent.

SIV and HIV gp120 molecules have a high amino acid sequence similarity, with about 40% amino acid sequence identity. In addition, the molecules have the same organization of variable and constant regions. The glycosylation sites in HIV and SIV gp120 are located in similar positions along the length of the molecules. Therefore, results from the SIV experiments described herein are believed to be applicable to preparing candidate HIV-1 vaccines. In fact, infection of rhesus monkeys with SIV is generally believed to be a useful model for assessing novel vaccine strategies for AIDS. See Wyand et al. 1996, *J. Virol.* 70:3724-3733 hereby incorporated by reference in its entirety.

EXAMPLE VII

In order to map the regions of the SIV envelope protein that can elicit antibodies in infected animals, reactivity against this region was tested with a panel of 23 peptides (as shown schematically in Fig. 15 which shows the amino acid sequence of SIVmac239, residues 89-213) in the following manner. Twenty-three biotinylated peptides were purchased from Chiron

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Mimotypes (Victoria, Australia) and bound to strept-avidin 96-well plates (Boehringer

Mannheim) over night at 4°C. Plates were washed 6 times in wash buffer (PBS and 0.1% Tween-

20) and animal sera was added at a 1:100 dilution for 90 minutes. Plates were washed and goat

anti-human antibody conjugated to alkaline phosphatase (AP; Kirkegaard and Perry) was added

5   for 90 minutes. Plated were washed again and an AP substrate (Kirkegaard and Perry) was

allowed to react with the enzyme at room temperature for 20 minutes.

Figs. 16-21 show the reactivity of sera with each peptide. Sera was from animals infected

with the indicated viruses for 16 weeks. Sera from week 0 was used as a negative control. As

the data indicates, in all cases, the viruses lacking the 4th glycosylation site elicited an immune

10   response against the corresponding peptide that spans the g4 site. However, the wild-type virus

was unable to elicit as strong an antibody response against this site. A similar response was

obtained with the antisera elicited by the viruses lacking the 5th or 6th glycosylation sites.

Consequently, removal of carbohydrates from the SIV envelope protein allows exposure of

previously unexposed antigenic sites. Fig. 22 shows the reactivity of all sera to peptide 14 which

15   contains an amino acid sequence which includes the 5th glycosylation site.

Dosage, Formulation and Mode of Administration

Vaccines comprising one or more HIV-1 gp120 molecules, as described herein, and

variants thereof having antigenic properties, can be prepared by procedures well-known in the art.

20   Procedures which are known for making wild-type envelope protein vaccines (e.g., such as those

produced by Chiron or Genentech) can be used to make vaccines with a selectively

underglycosylated envelope protein of the invention. Various modifications such as adjuvants and

other viral or toxin components known for such vaccines or immunotherapeutics may be

incorporated with the mutant molecule. For examples, such vaccines may be prepared as

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injectables, e.g., liquid solutions or suspensions. Solid forms for solution in or suspension in a liquid prior to injection also can be prepared. Optionally, the preparation also can be emulsified. The active antigenic ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Examples of suitable excipients are water,

5   saline, dextrose, glycerol, ethanol, etc., and combinations thereof. In addition, if desired, the vaccine can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants, such as aluminum hydrazide or muramyl dipeptide, which enhance the effectiveness of the vaccine. The vaccines are conventionally administered parenterally, by injection, for example either subcutaneously or intramuscularly. Additional formulations which

10   are suitable for other modes of administration include suppositories and in some case oral formulations.

The peptides or proteins can be formulated into a vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example,

15   hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups also can be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium , or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

In accordance with another aspect of the invention, there is provided a method for

20   immunizing an animal comprising the steps of obtaining a preparation comprising an expressible DNA coding for

recombinant immunogenic HIV-1 envelope mutant glycoprotein gp120 or gp160 molecule, and introducing the preparation into an animal wherein the translation product of the DNA is formed by a cell of the animal, which elicits an immune response against the immunogen. Further

32

vaccines may be prepared using a live virus approach well known in the art. The injectable

preparation comprises a pharmaceutically acceptable carrier containing an expressible DNA

coding the immunogenic HIV-1 envelope mutant glycoprotein gp120 or gp160 molecule or the

live virus containing the DNA coding the immunogenic HIV-1 envelope mutant glycoprotein

5   gp120 or gp160 molecule, and on the introduction of the preparation into the animal, the

polynucleotide or live virus is incorporated into a cell of the animal wherein an immunogenic

translation product of the DNA is formed, which elicits an immune response against the

immunogen. In an alternative embodiment, the preparation comprises one or more cells obtained

from the animal and transfected in vitro with the DNA, whereby the DNA is incorporated into the

10   cells, where an immunogenic translation product of the DNA is formed, and whereby on the

introduction of the preparation into the animal, an immune response against the immunogen is

elicited. The polynucleotide used for immunization may be an mRNA sequence, although a non-

replicating DNA sequence may be used. The DNA may be introduced into the tissues of the body

using the injectable carrier alone; liposomal preparations are preferred for methods in which in

15   vitro transfactions of cells obtained from the animal are carried out. The carrier is preferably

isotonic, hypotonic or weakly hypertonic, and has a relatively low ionic strength, such as provided

by a sucrose solution.

The vaccines are administered in a manner compatible with dosage formulation an in such

amount as will be therapeutically effective and immunogenic. The quantity to be administered

20   depends on the subject to be treated, capacity of the subject's immune system to synthesize

antibodies, and the degree of protection desired. Precise amounts of active ingredient required

to be administered depend on the judgment of the practitioner and are peculiar to each individual.

However, suitable dosage ranges are of the order of about several hundred micrograms active

ingredient per individual. Suitable regimes for initial administration and booster shots are also

variable, but are typified by an initial administration followed in one or two week intervals by a

subsequent injection or other administration. The efficacy of a vaccine according to the invention

may be determined based on any clinical parameter which a medical doctor assesses for

determining the onset and progress of HIV-1 infection or for determining whether an individual

5   has AIDS. Such parameters include, for example, measuring the level of T-cells in a patient.

Acceptable levels of T-cells in an uninfected patient are in the range of 1000-2000 T cells per

mm^3.

It is to be understood that the embodiments of the present invention which have been

described are merely illustrative of some of the applications of the principles of the invention.

10   Numerous modifications may be made by those skilled in the art based upon the teachings

presented herein without departing from the true spirit and scope of the invention.

What is claimed is:

1.   A composition comprising a recombinant human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein having an amino acid sequence which is altered with respect to a wild type HIV-1 envelope glycoprotein, said altered amino acid sequence including a mutated

5   consensus amino acid recognition sequence for N-linked carbohydrate attachment which as a result of said mutated consensus amino acid recognition sequence is not glycosylated in a mammalian host cell, said mutated consensus amino acid recognition sequence being positioned between the N-terminus of gp120 and the Cysteine at the N-terminal side of the gp120 cysteine V3 loop, said Cysteine being approximately at amino acid position 296, said recombinant envelope

10   glycoprotein having a mutated consensus amino acid recognition sequence for N-linked carbohydrate attachment being effective, when present as a component of a complete HIV virion, to support viral infectivity.

2.   The composition of claim 1, wherein said wild type HIV-1 envelope glycoprotein

15   is gp160 or a fragment thereof.

3.   The composition of claim 1, wherein said mutated consensus amino acid recognition sequence for N-linked carbohydrate attachment contains a substitution of Asn, Ser, or Thr as it occurs in the natural consensus sequence with a different amino acid.

20

4.   The composition of claim 1 wherein there is a mutated consensus amino acid sequence at an N-linked carbohydrate attachment site in the region between the N terminus of gp120 and the Cys on the C-terminal side of the cysteine loop containing hypervariable region 2 (V2).

5.   The composition of claim 1 wherein there are multiple mutated consensus amino acid sequences at N-linked carbohydrate attachment sites in said region.

6.   A vaccine for use in protection of a human against infection with HIV-1, said

5   vaccine comprising the composition of claim 1.

7.   A vaccine for use in treatment of a human infected with HIV-1, said vaccine comprising the composition of one of claim 1.

10   8.   The vaccine of claim 6 or 7, comprising an HIV-1 virion containing a gp120 molecule having an altered amino acid sequence comprising a mutated consensus amino acid recognition sequence for N-linked carbohydrate attachment which as a result of said mutated consensus amino acid recognition sequence is not glycosylated in a mammalian host cell, said mutated consensus amino acid recognition sequence being positioned between the N-terminus of

15   gp120 and the Cysteine at the N-terminal side of the gp120 cysteine V3 loop, said Cysteine being approximately at amino acid position 296, said HIV-1 virion being infective.

9.   Antibodies to the composition of claim 1 produced by challenging a mammal with said composition.

20

10.   The antibodies of claim 9 wherein said antibodies are monoclonal antibodies.

11.   A method of inducing a protective immune response in a mammal comprising administering to the mammal an effective amount of the composition of claim 1 or the vaccine of

any one of claims 6-8 sufficient to induce the protective immune response. prevent or delay

infection by HIV-1.

12.   A method of vaccinating a patient against HIV-1 comprising administering to the

patient an effective amount of the composition of claim 1 or the vaccine of any one of claims 6-8

sufficient to prevent or delay infection by HIV-1.

Fig. 1

Fig. 2

V0 V1 V2 V3 V4 V5

SIVmac239 114 NKSETDRWGLTKSITTTASTTSTTASAKVDMVNETSSCIAQDNCT 158
g4
g5
g6
g45
g46
g56
g456
M144V
g456MV
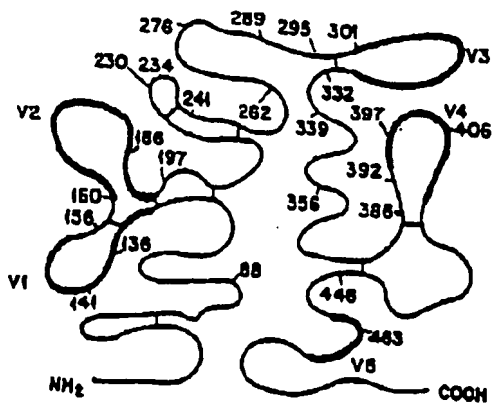
Fig. 3

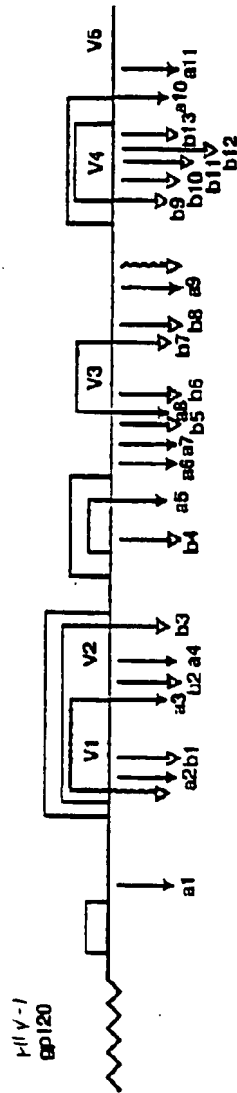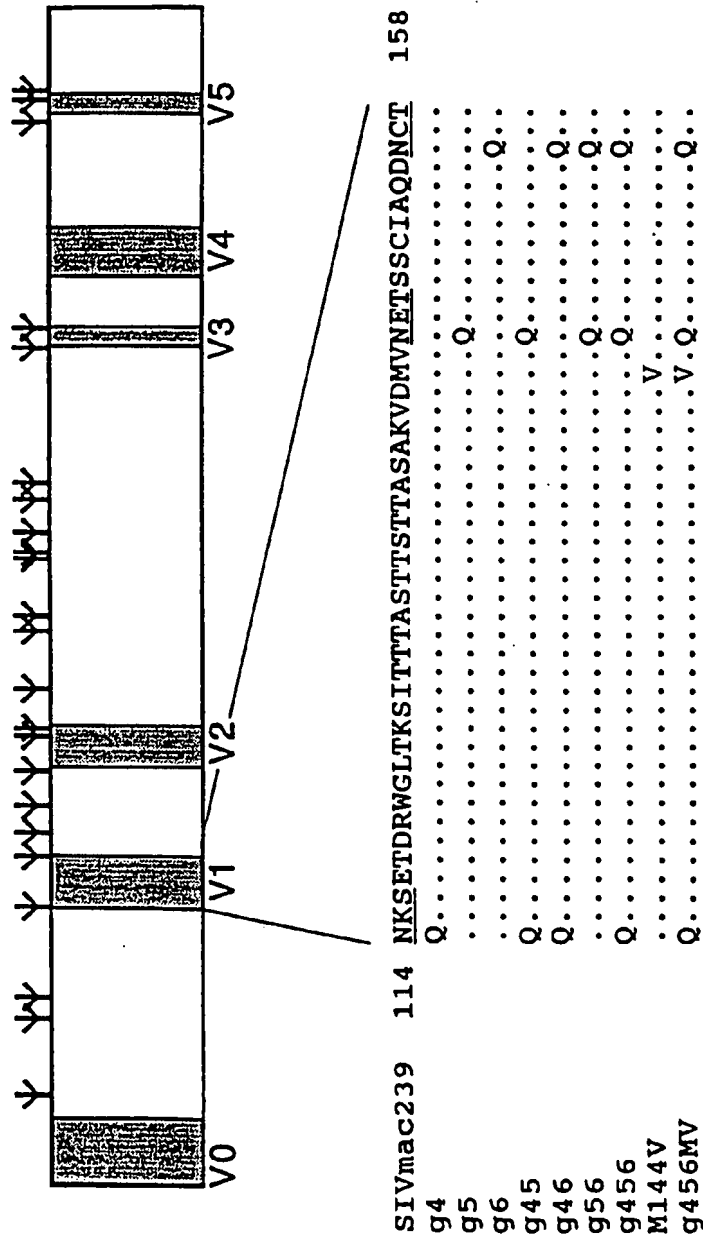Infection of CEMx174 Cells by SIV239mac Mutant Viruses
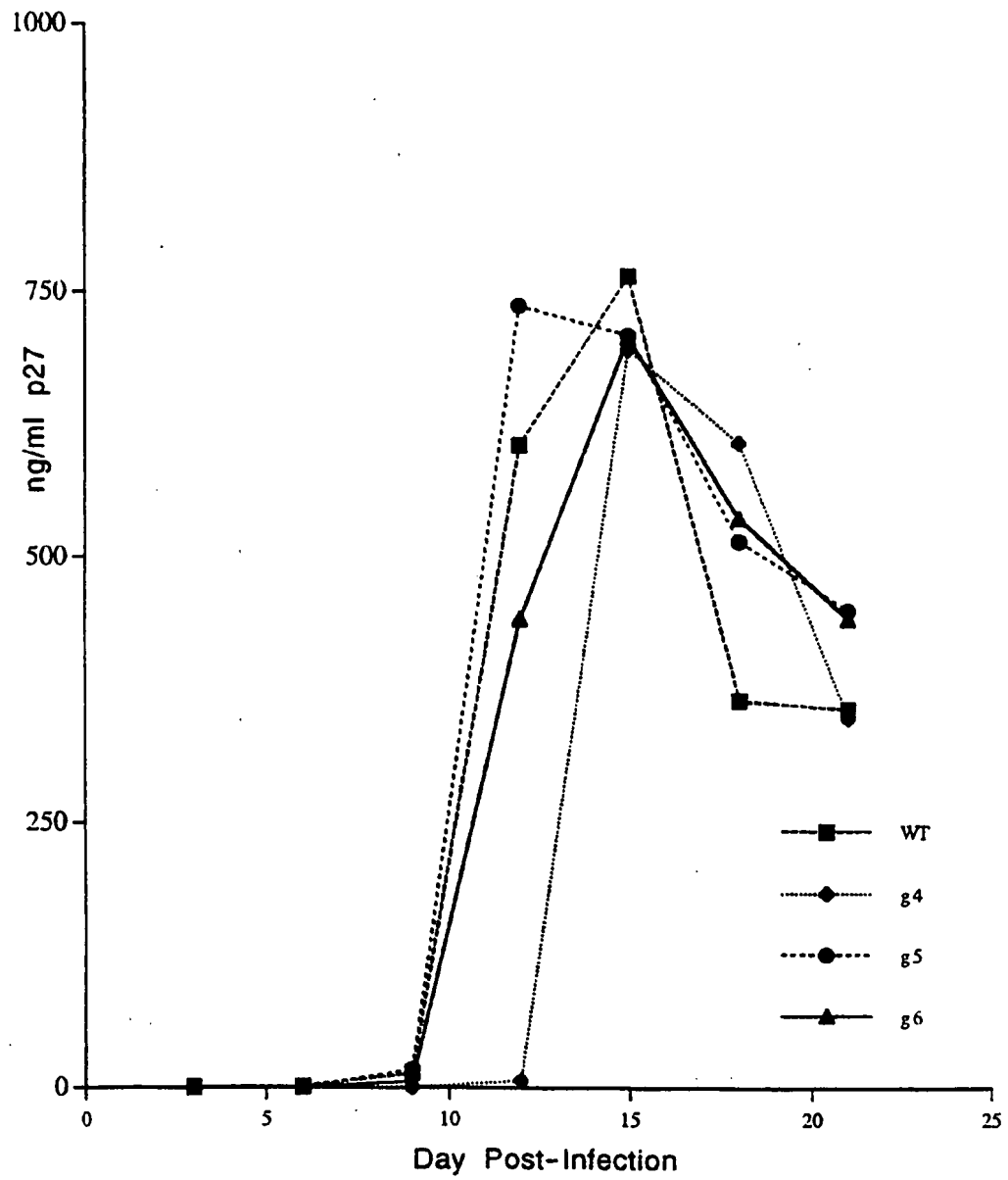With Single Glycosylation Substitutions in *env*

Fig. 4

Infection of CEMx174 Cells by SIV239mac Mutant Viruses
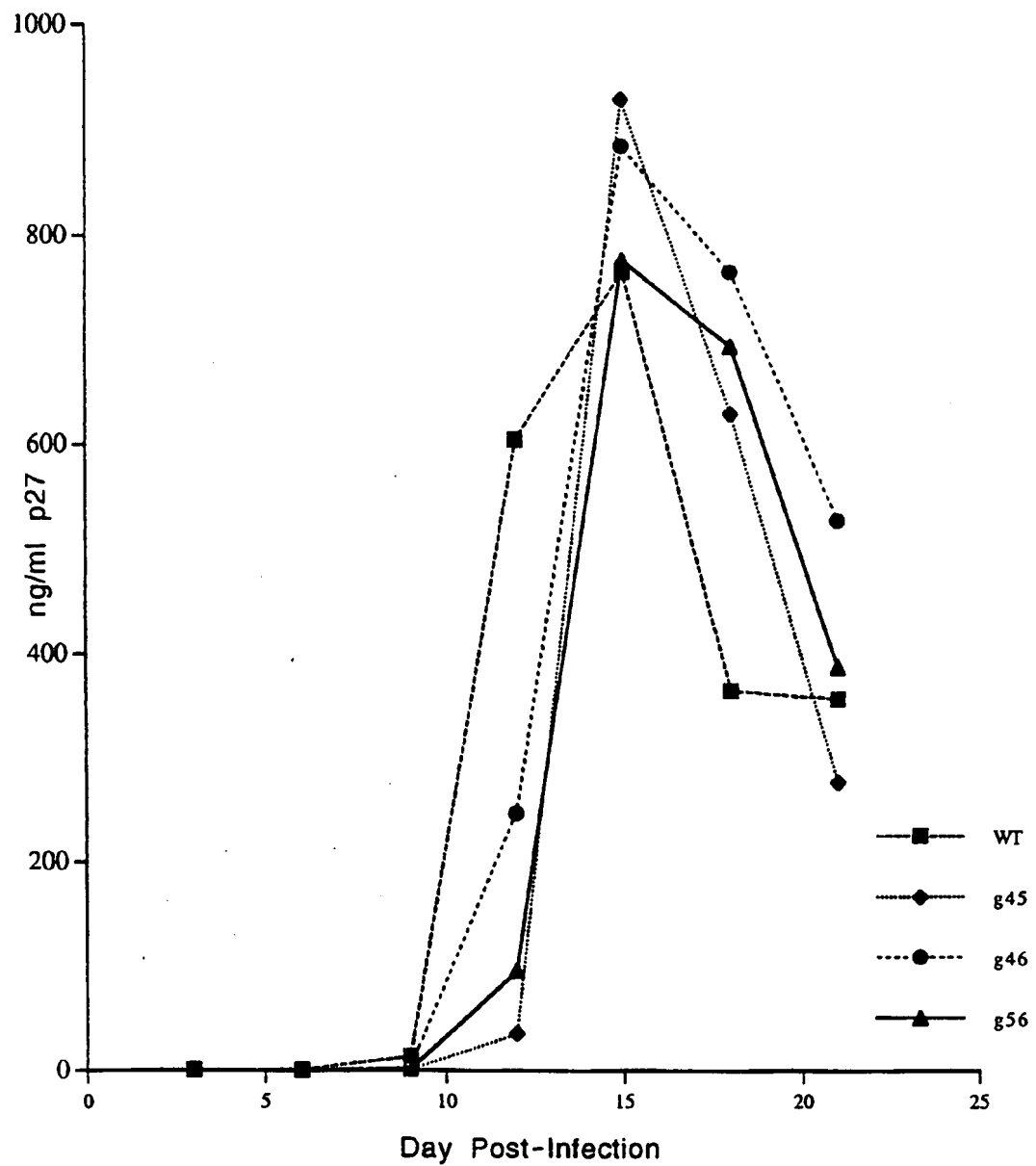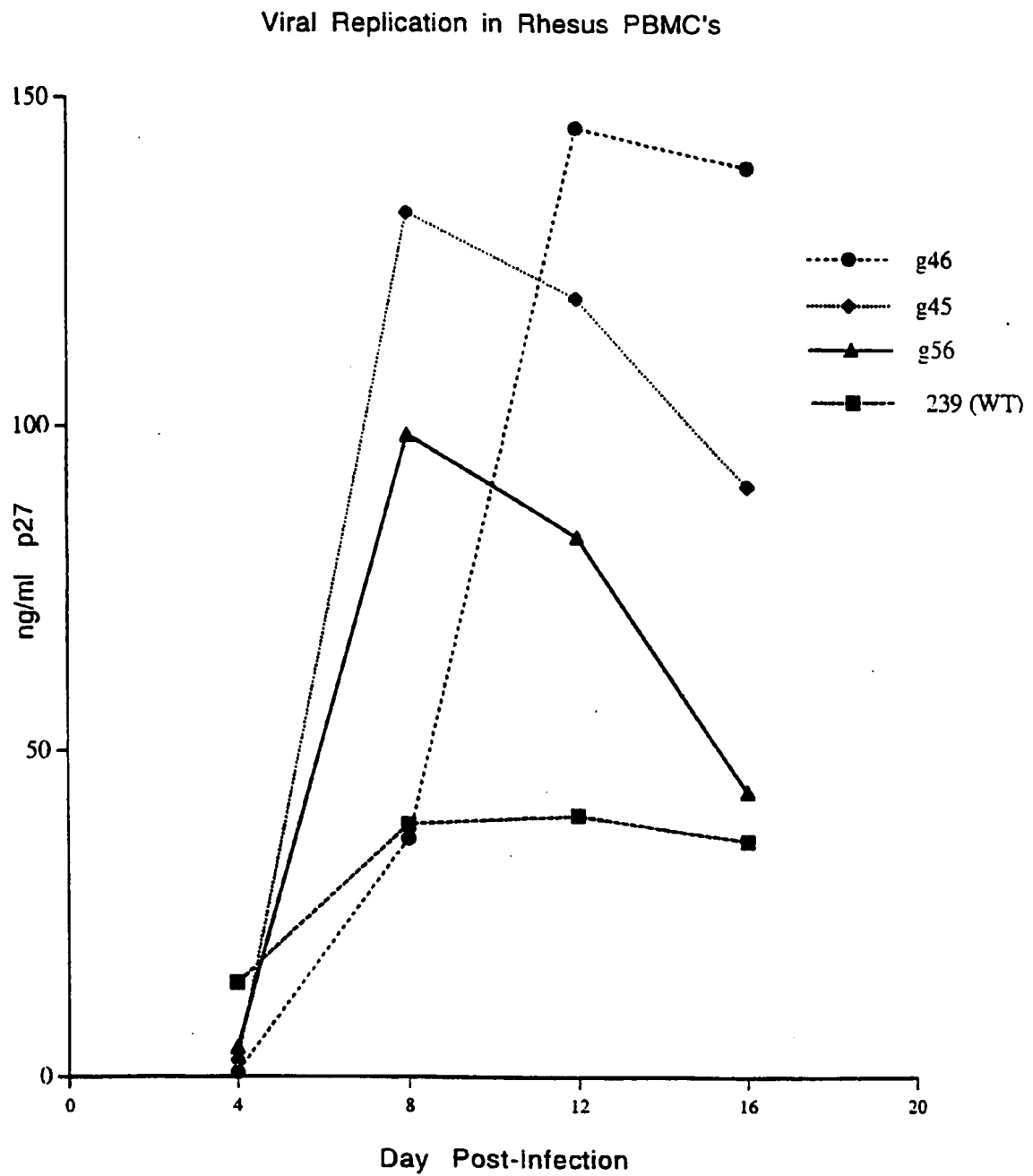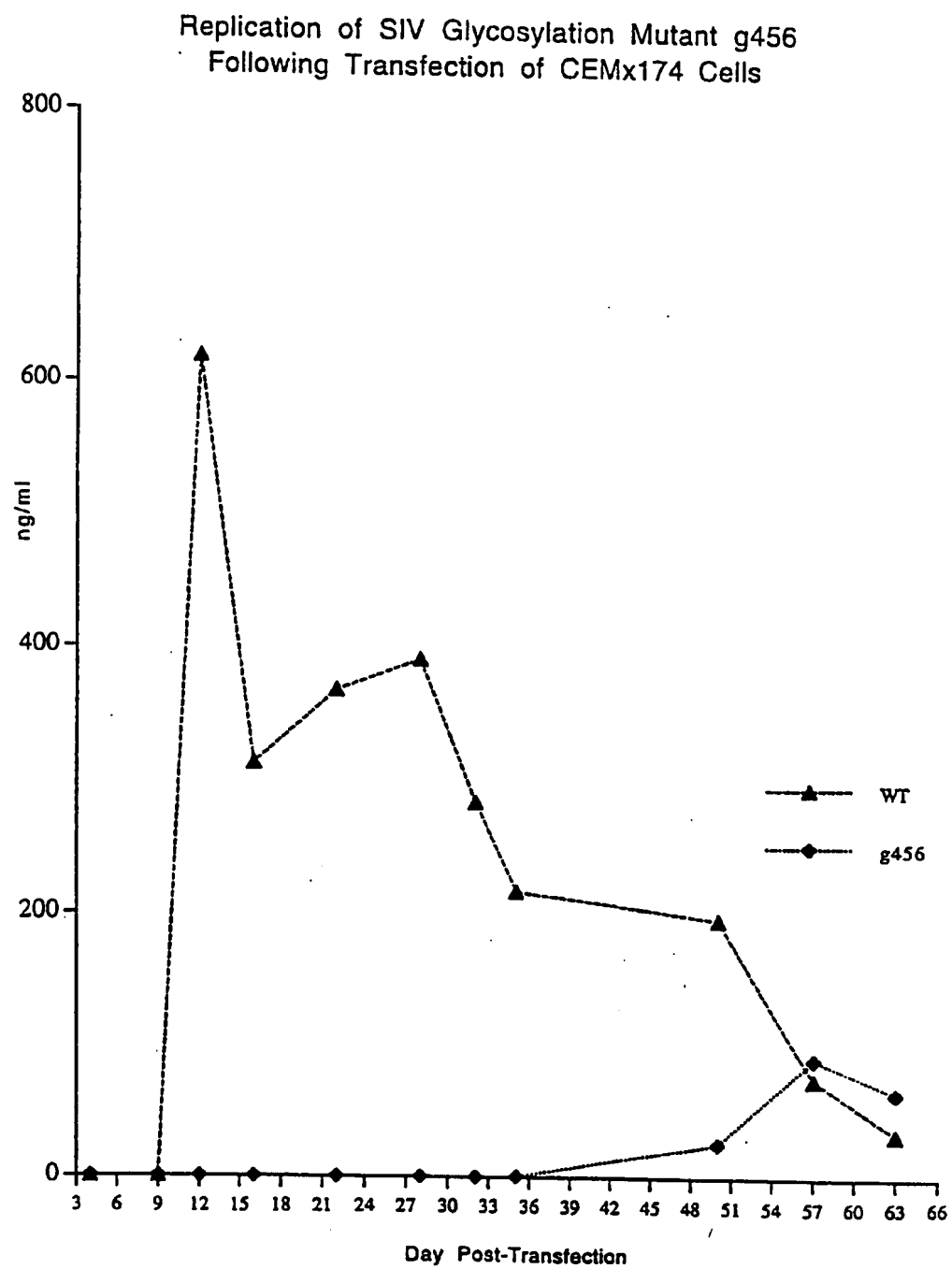With Multiple Glycosylation Substitutions in *env*

Fig. 5

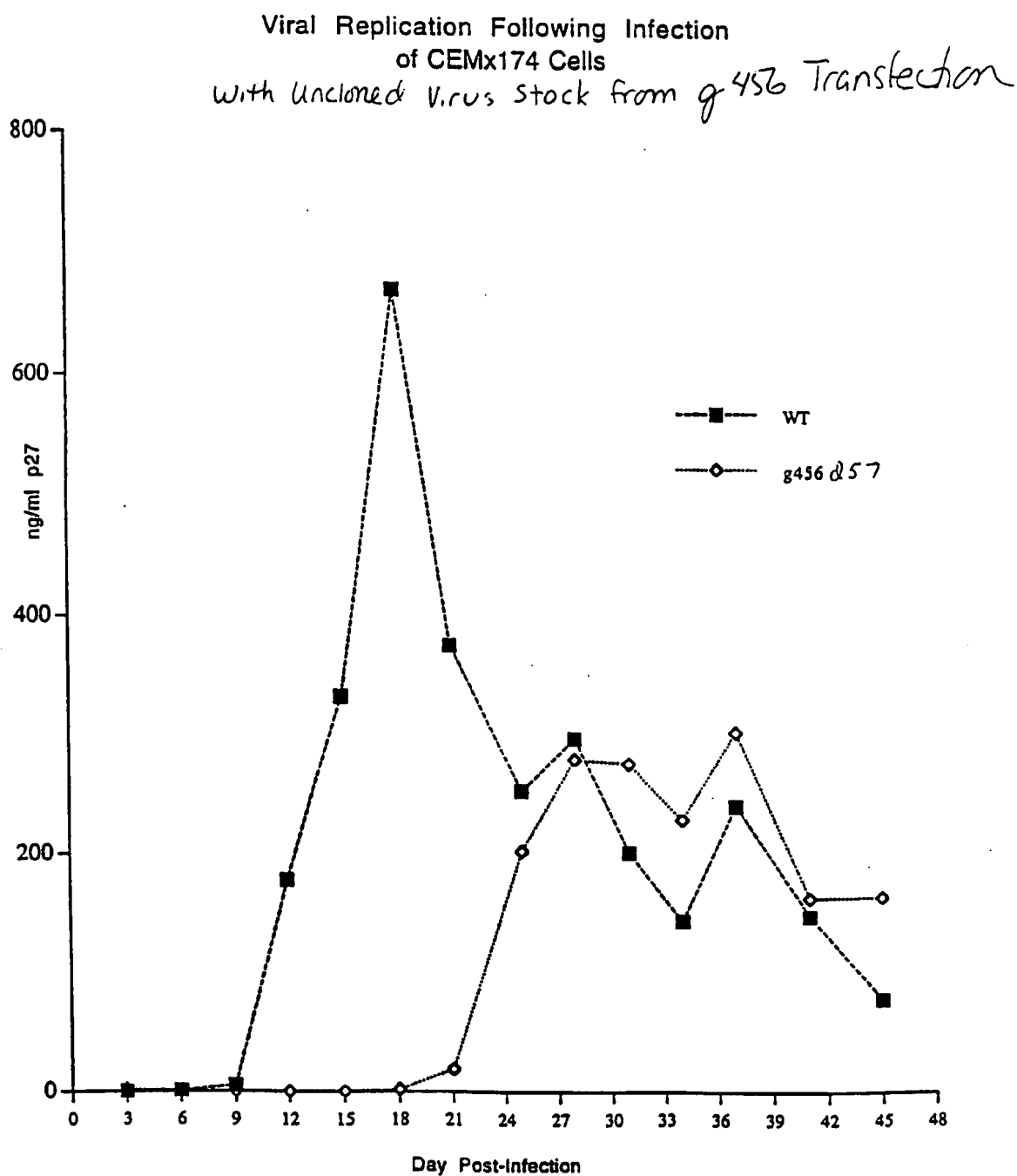## Viral Replication in Rhesus PBMC's

Fig. 6

Fig. 7

Viral Replication Following Infection
of CEMx174 Cells
with Uncloned Virus Stock from g456 Transfection

Fig. 8

Sequence of g456 Revertant Clones

```
              10          20          30          40          50          60
clr  H G C L G H Q L L I A I L L L S V Y G I Y C T L Y V T V F Y G V P A W R N A T I P L F C A T K N R D T W G T T Q C L P D H G
g456P . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R7 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R9 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


              70          80          90          100         110         120
     D Y S E V A L N V T E S F D A W N N T V T E Q A I E D V W Q L F E T S I K P C V K L S P L C I T M R C Q K S E T D R W G L T
g456R1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R7 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R9 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


              130         140         150         160         170         180
     K S I T T T A S T T S T T A S A K V D H V Q E T S S C I A Q D Q C T G L E Q E Q M I S C K F N H T G L K R D K K K E Y N E T
g456R1 . . . . . . . . . . . . . . . . . . . V . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g  R7 . . . . . . . . . . . . . . . . . . . V . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
gr R9 . . . . . . . . . . . . . . . . . . . V . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


              190         200         210         220         230         240
     W Y S A D L V C E Q G N H T G N E S R C Y M N H C N T S V I Q E S C D K H Y W D A I R F R Y C A P P G Y A L L R C N D T N
g456R1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R7 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . V . . . . . . . . . . . . . . . . . . . .
g456R9 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . a . . . . . . . . . . . . . . . . . . . .


              250         260         270         280         290         300         310
     Y S C F M P E C S K V V V S S C T R M M E T Q T S T W F G F N G T R A E N R T Y I Y W H G R F H R T I I S L N K Y Y N L T M
g456R1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R7 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R9 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


              320         330         340         350         360         370
     K C R R P G N K T V L P V T I M S G L V F H S Q P I N D R P K Q A W C W F G G K W K D A I K E V K Q T I V K H P R Y T G T N
g456R1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R7 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R9 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


              380         390         400         410         420         430
     N D K I H L T A P G G G D P E V T P H W T N C R G E F L Y C K M H W F L H V V E D R H T A H Q K P K E Q H K R H Y V P C H I
g456R1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R7 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R9 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .


              440         450         460         470         480         490
     R Q I I N T W H K V G K N V Y L P P R E G D L T C N S T V T S L I A N I D W I D G N Q T N I T I S A E V A E L Y R L E L G D Y
g456R1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R7 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
g456R9 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
```

Fig. 9
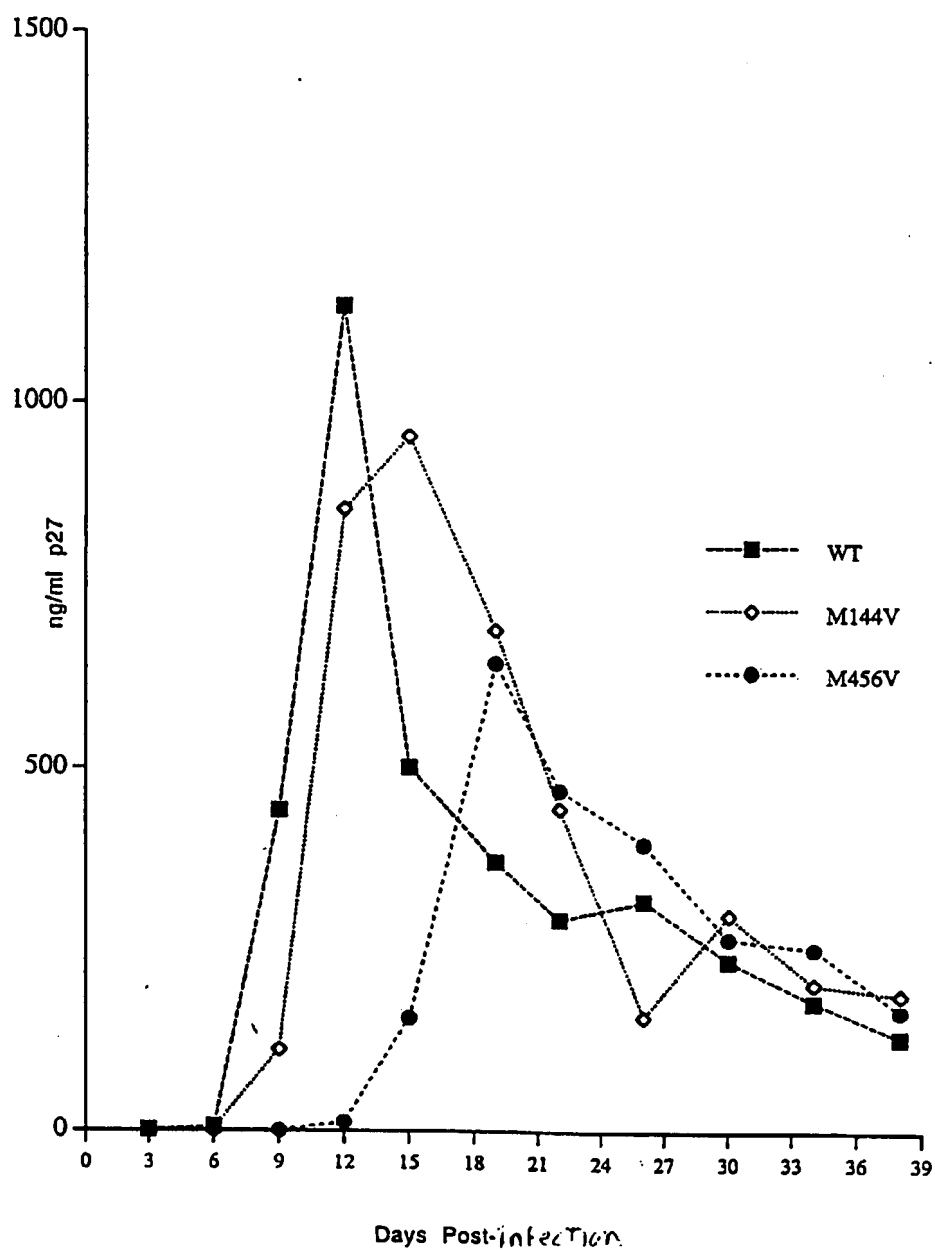
Viral Replication Following Infection
of CEMx174 Cells

Fig. 10
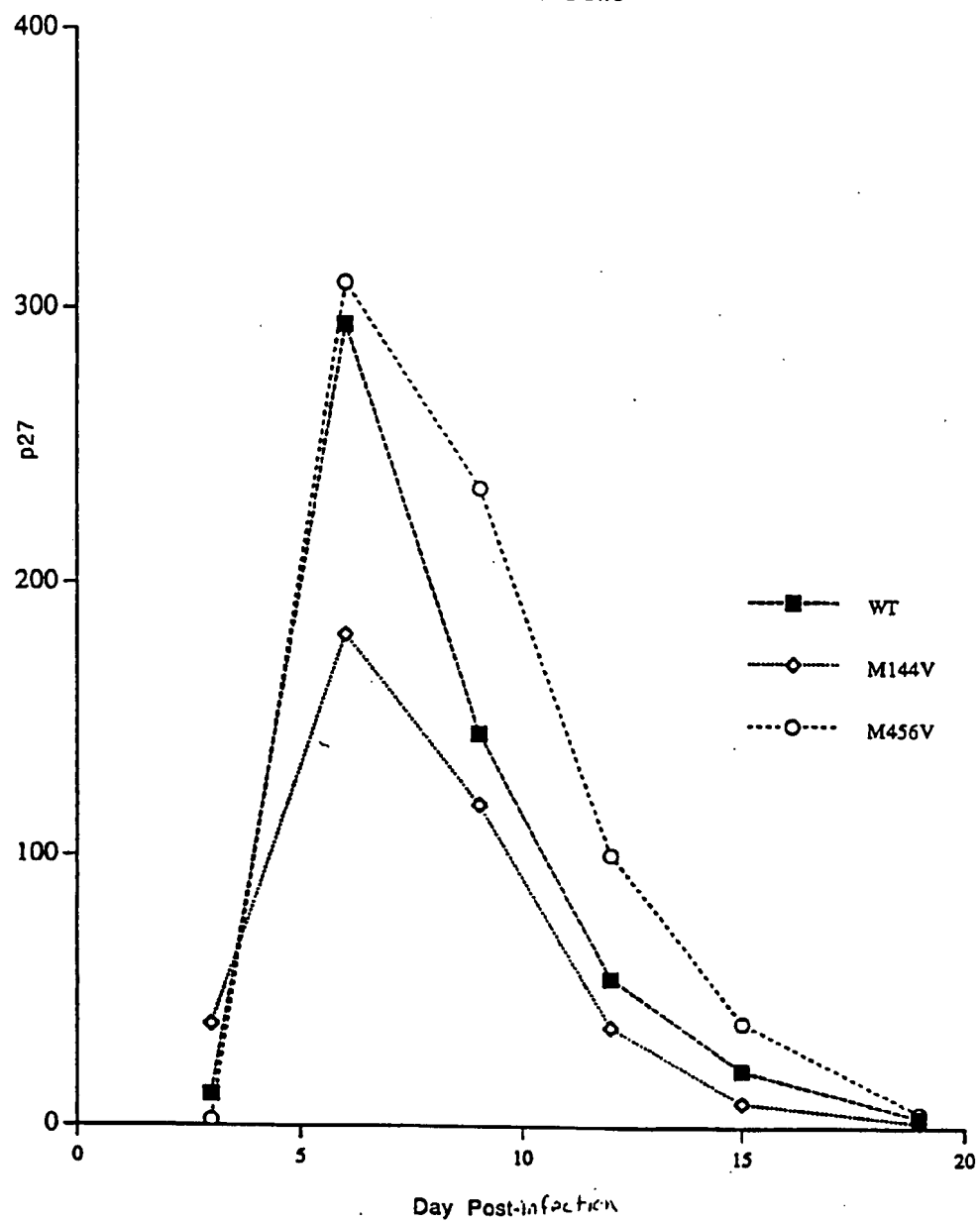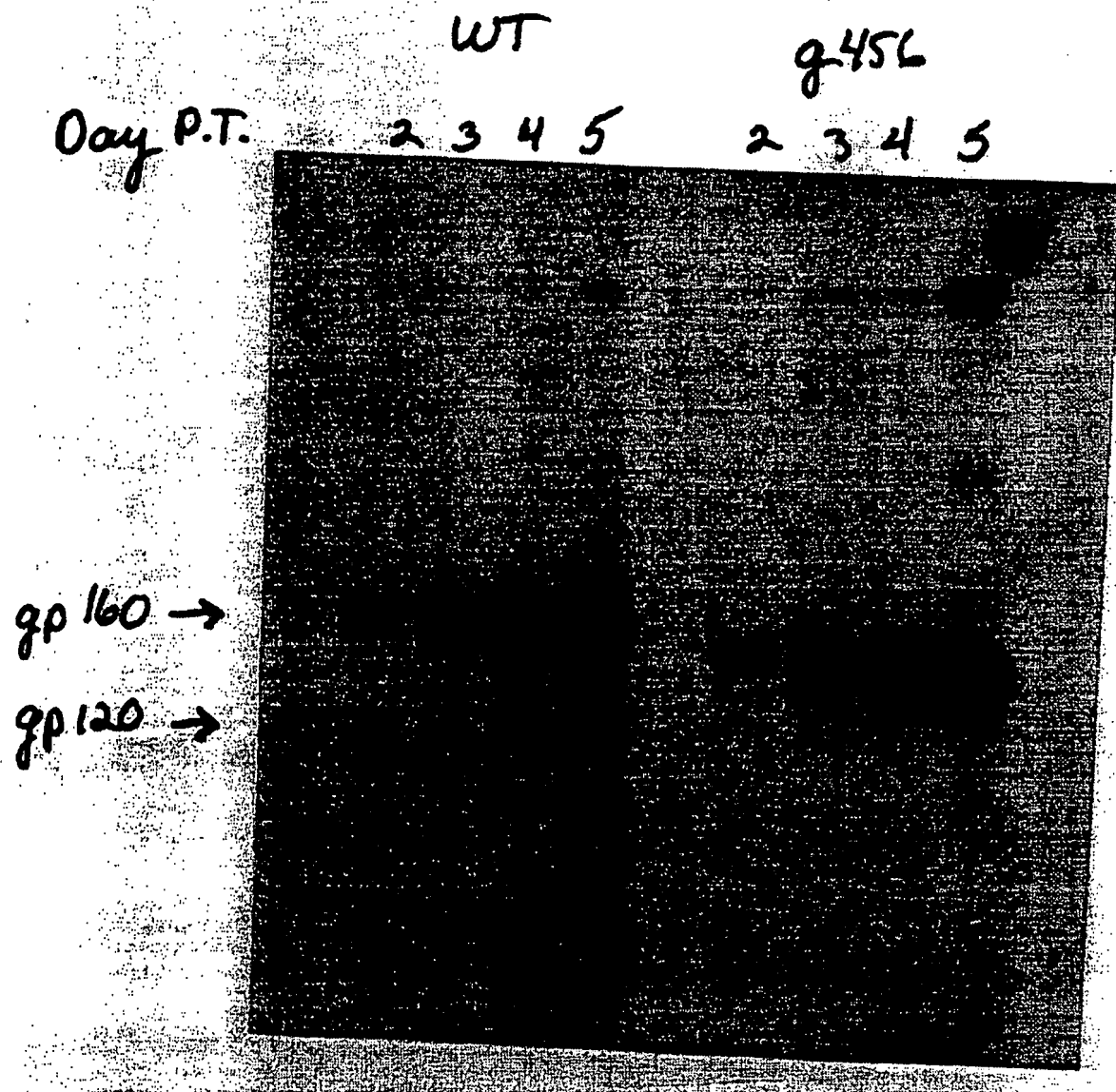
Fig. 11

Fig. 12

Fig. 13

Antibody Responses to Peptide at 6 months P.I.

Fig. 14

```
          90        100       110       120       130
 1  DVWQLFEETSIKPCVKLSPLCITMRCNKSETDRWGLTKSITTTASTTSTT
 2              SIKPCVKLSPLCI
 3                CVKLSPLCITMRC
 4                  SPLCITMRCNKSE
 5                      ITMRCNKSETDRW
 6                        CNKSETDRWGL
 7                            ETDRWGLTKSITT
 8                                WGLTKSITTTAST
 9                                  KSITTTASTTSTT

          130       140       150       160       170
10  TTASTTSTTASAKVDMVNETSSCIAQDNCTGLEQEQMISCKFNMTGLKR
11              TTSTTASAKVDMV
12                TASAKVDMVNETS
13                  KVDMVNETSSCIA
14                      VNETSSCIAQDNC
15                        SSCIAQDNCTGLE
16                            AQDNCTGLEQEQM
17                              CTGLEQEQMISCKF
18                                EQEQMISCKFNMTG

          170       180       190       200       210
19  CKFNMTGLKRDKKKEYNETWYSADLVCEQGNNTGNESRCYMNHCNT
20              LKRDKKKEYNETWY
21                EYNETWYSADLVCE
22                    SADLVCEQGNNTGN
23                      QGNNTGNESRCYMN
```
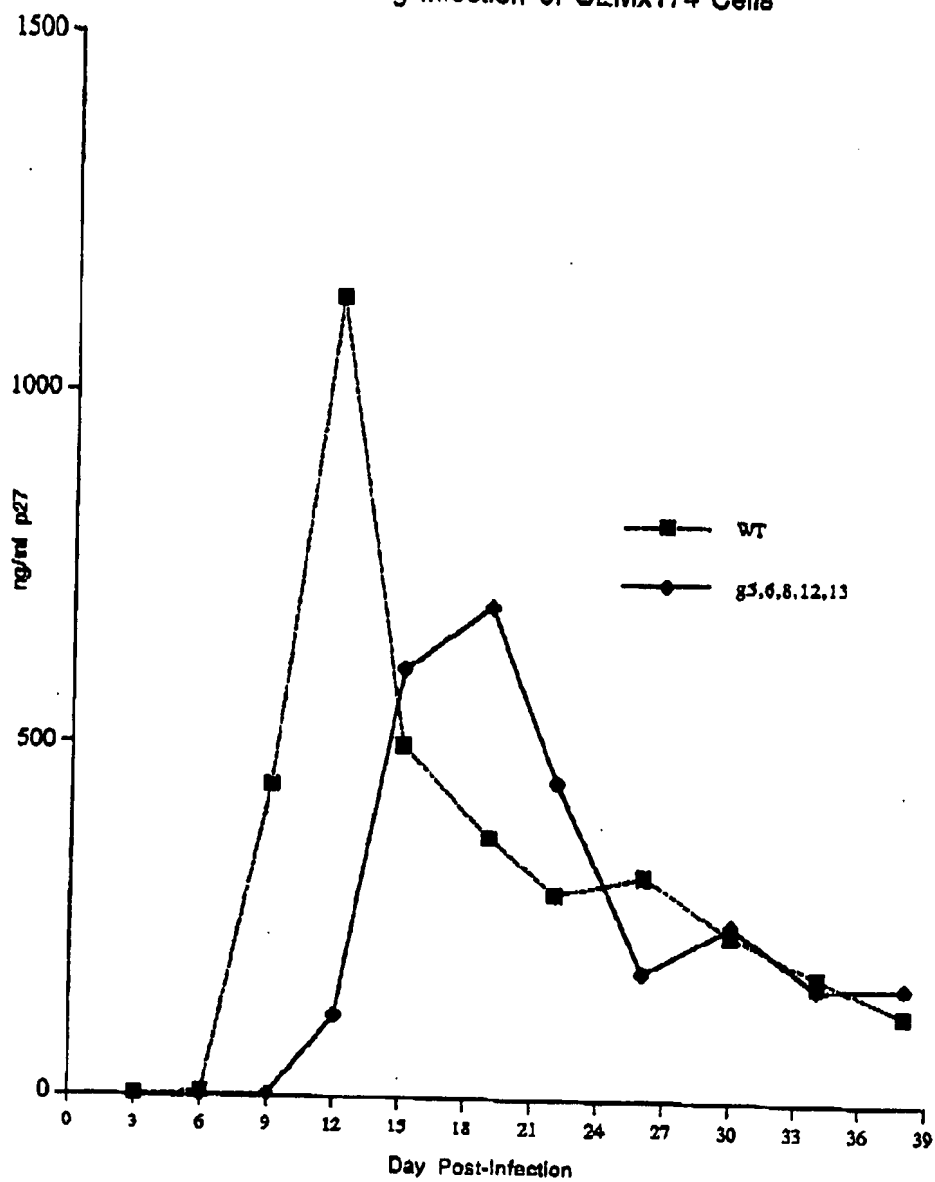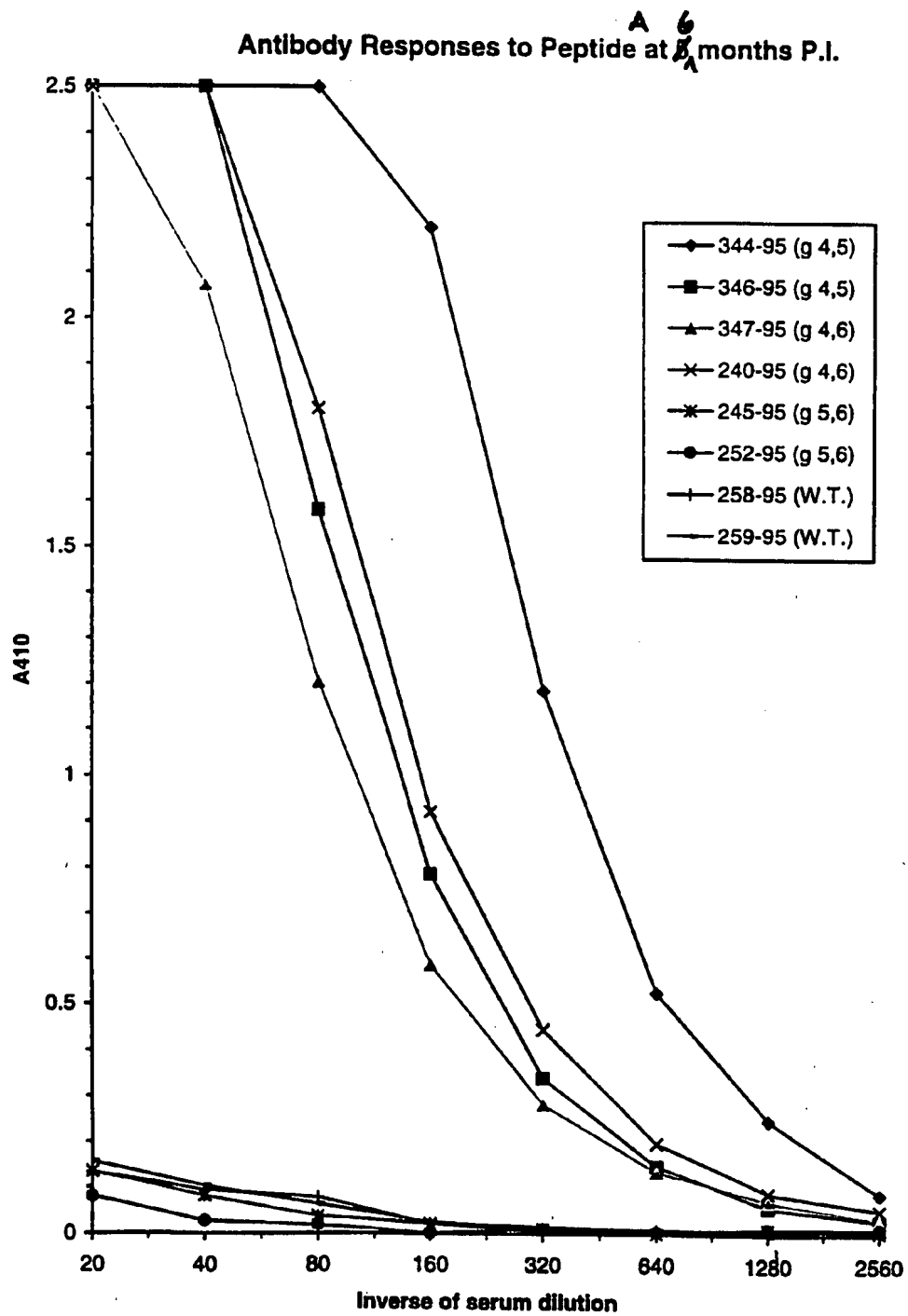
Fig. 15

Reactivity of Animal Sera With Serial Peptides
WT #259 and g46 #346
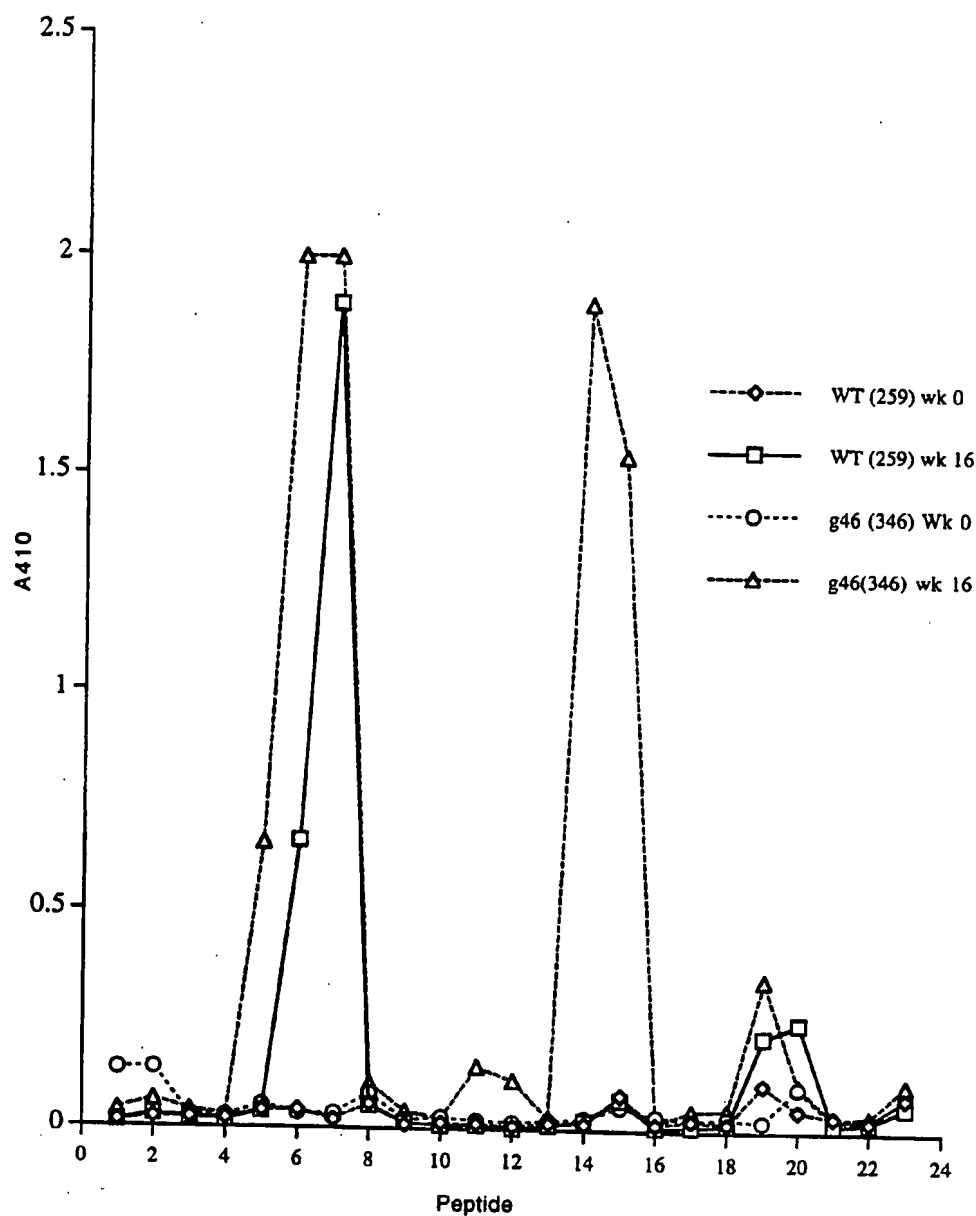
Fig. 16

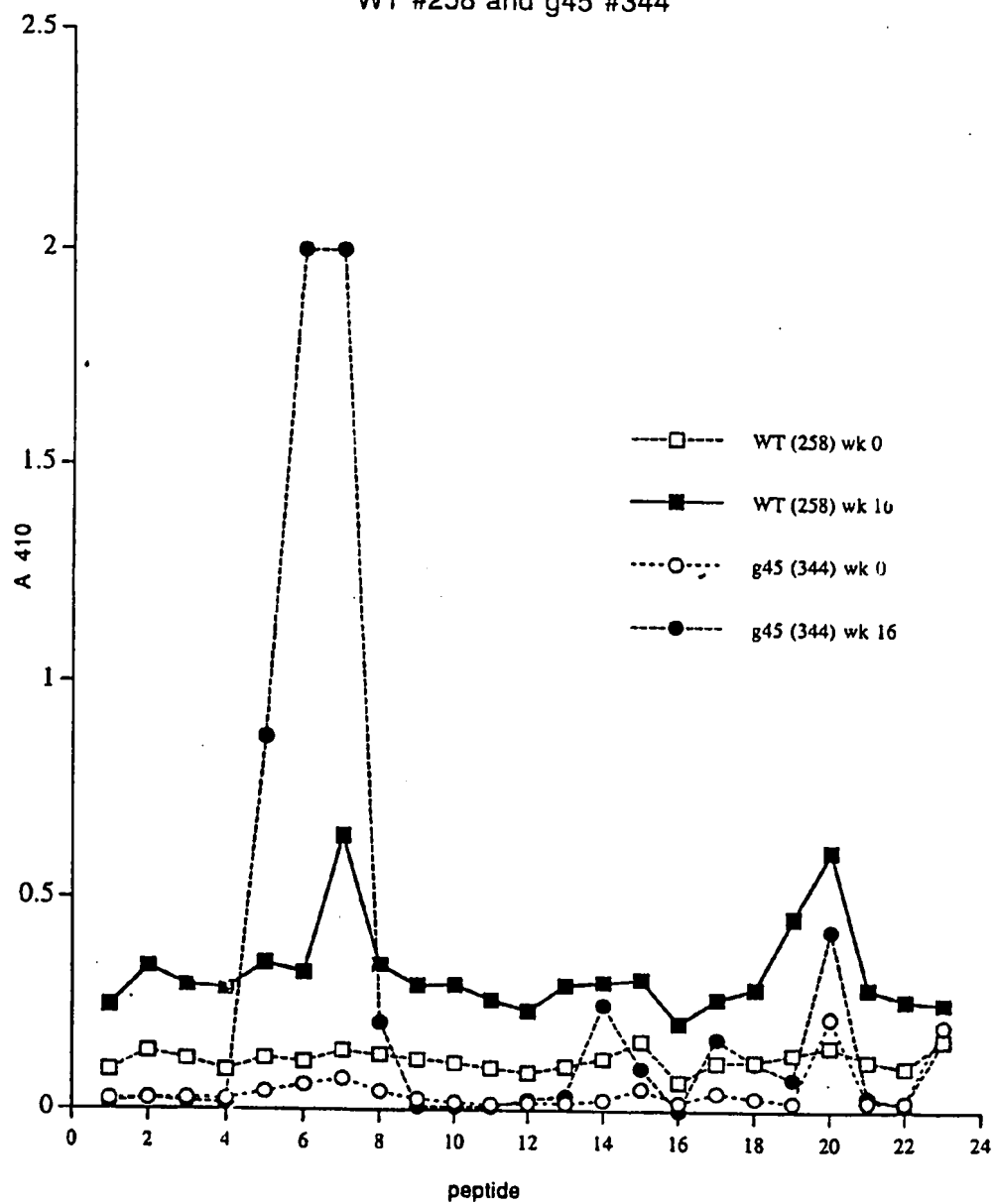Reactivity of Animal Sera With Peptide Series
WT #258 and g45 #344

Fig. 17

Reactivity of Animal Sera With Peptide Series
g56 #252 and g45 #347
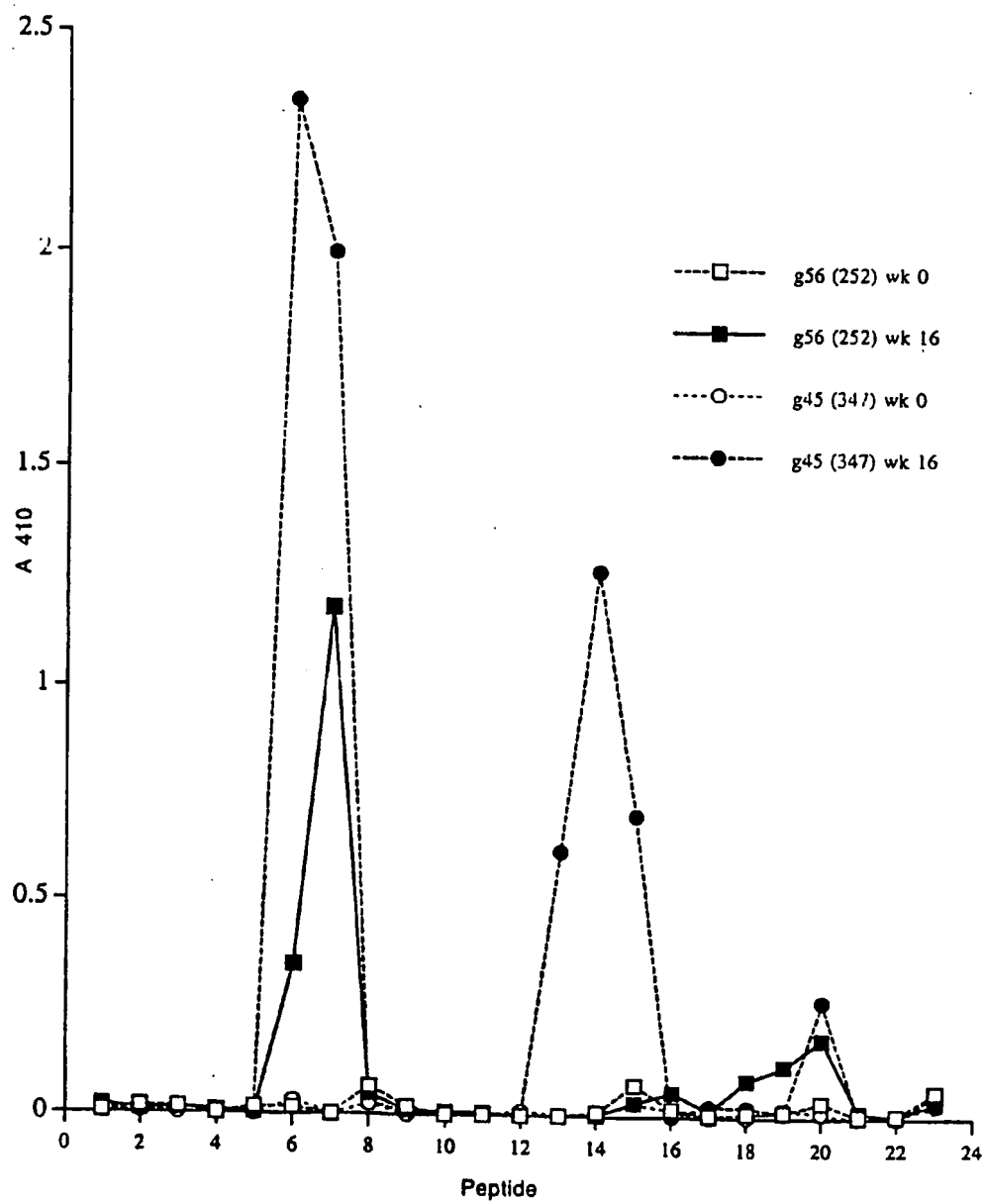
Fig. 18

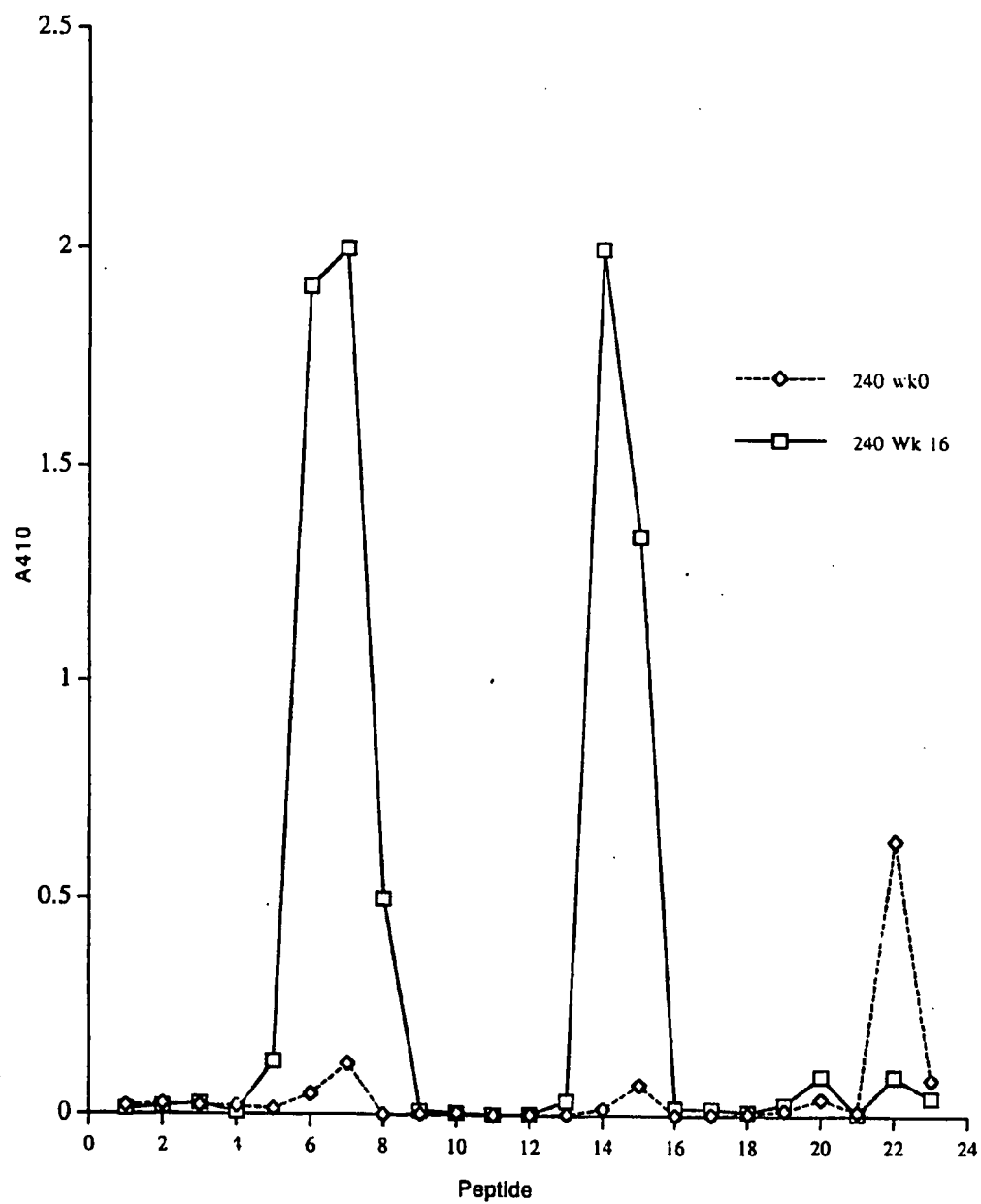Reactivity of Sera With Peptide Series
Animal #240 Infected with g46

Fig. 19

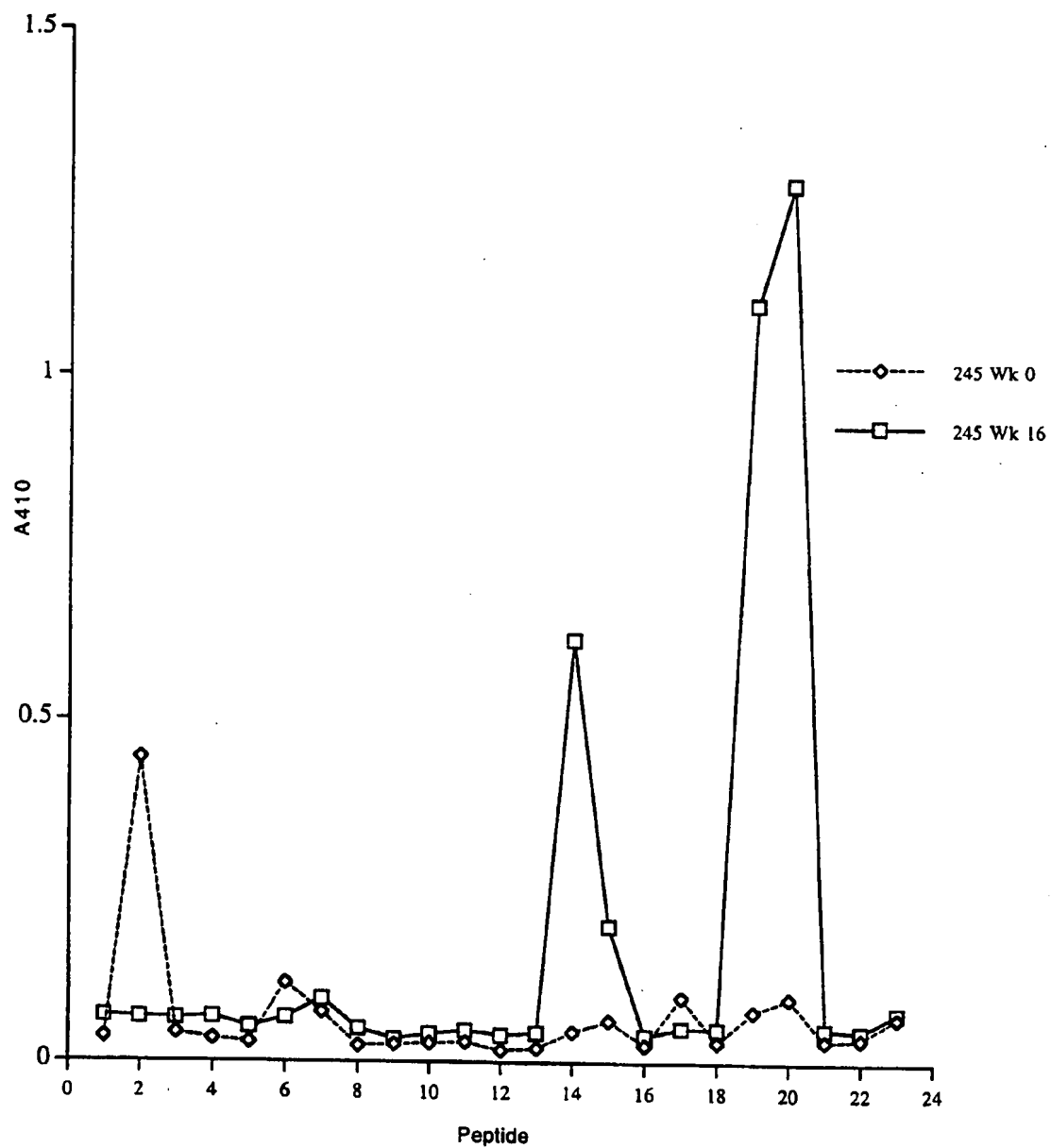Reactivity of Sera With Peptide Series
Animal #245 Infected With g56

Fig. 20

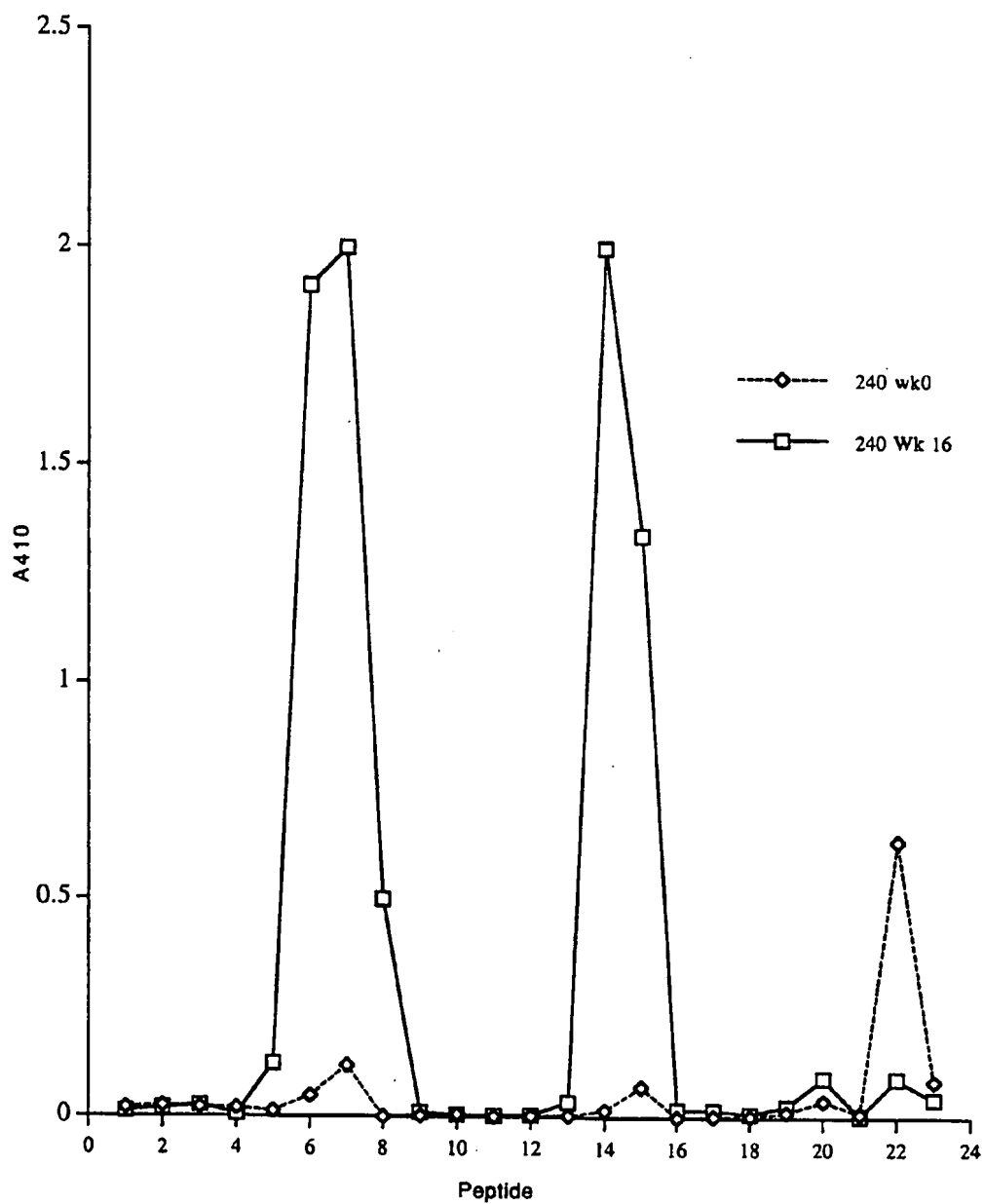Reactivity of Sera With Peptide Series
Animal #240 Infected with g46

Fig. 21

Animal Sera Antibody Responses to Peptide 14
Following 24 Weeks Infection With Mutant
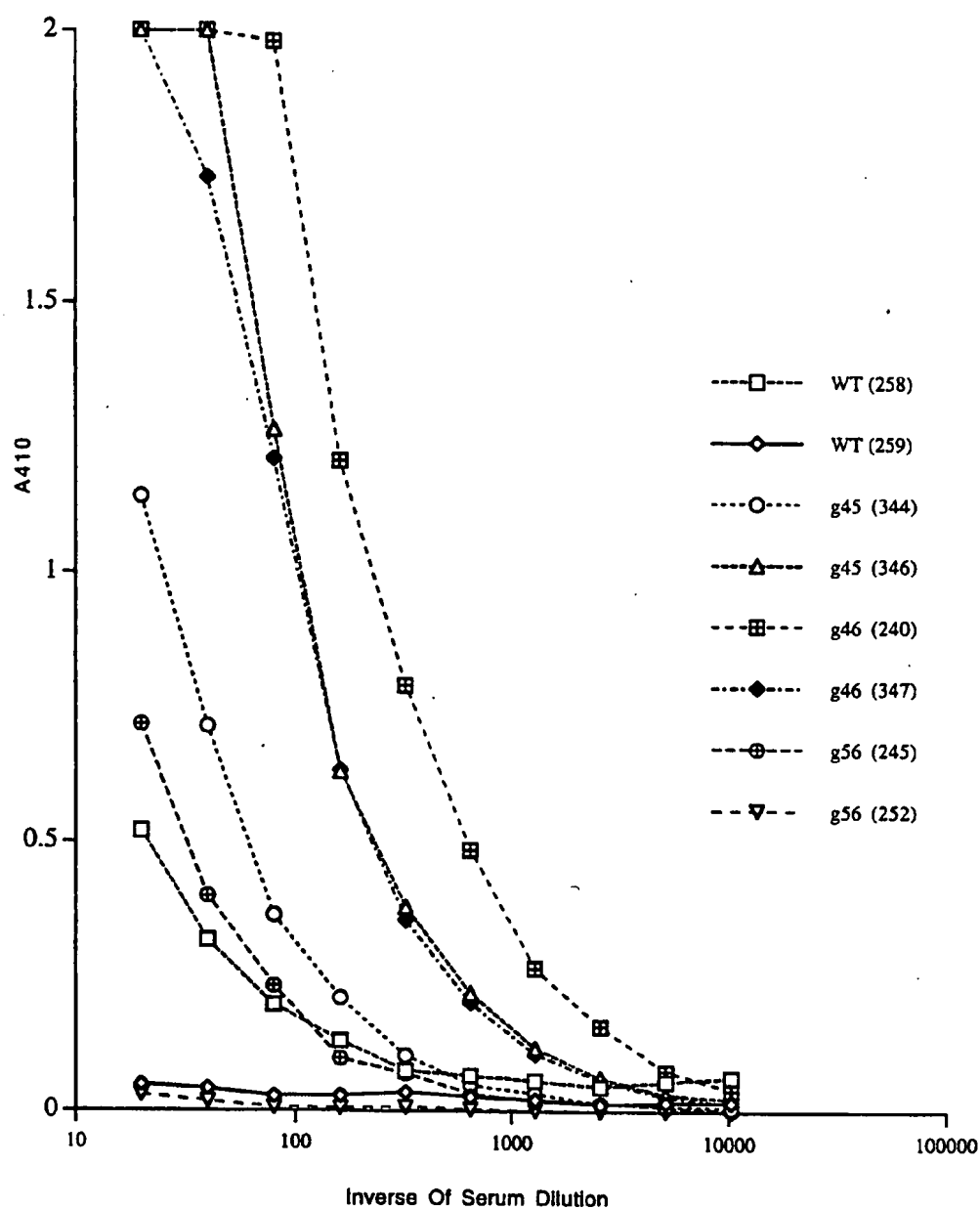and Wild-Type SIVmac239 Viruses

Fig. 22

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :CO7K 1/00, 14/00, 17/00; A61K 39/00, 39/38, 39/21
US CL : 530/350, 351; 424/184.1, 188.1, 204.1, 208.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 351; 424/184.1, 188.1, 204.1, 208.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog
Search terms: HIV, envelope glycoprotein, mutations, N-linked glycosylation, glycans, composition

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Derwent WPI on Dialog, Derwent Into Ltd., Accession No.93-303140/199338, WO 9317705 A1 (ESSEX et al) 16 September 1993 (09/16/93), abstract.	1-5
Y	LEE, W. et al. Nonrandom Distribution of gp120 N-linked Glycosylation Sites Important for Infectivity of Human Immunodeficiency Virus Type 1. Proceedings National Academy of Sciences, USA. March 1992, pages 2213-2217, especially pages 2213-2216.	1-5, 9, 10
Y	BACK et al. An N-Glycan Within the Human Immunodeficiency Virus Type 1 gp120 V3 Loop Affects Virus Neutralization. Virology. 1994, Vol 199, pages 431-438, see entire document.	1-5, 9, 10

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 JUNE 1998	27 JUL 1998

Name and mailing address of the ISA/US	Authorized Officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	LYNETTE F. SMITH
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DOE et al. Induction of HIV-1 Envelope (gp120)-specific Cytotoxic T Lymphocyte Responses In Mice By Recombinant CHO-Cell-Derived gp120 Is Enhanced by Enzymatic Removal of N-Linked Glycans. European Journal of Immunology. 1994, Vol 24, pages 2369-2376, see entire document.	1-5, 9, 10
Y	BOTARELLI et al. N-Glycosylation of HIV-gp120 May Constrain Recognition By T Lymphocytes. The Journal of Immunology. 01 November, 1991, Vol 147. No. 9. pages 3128-3132, see entire document.	1-5, 9, 10
Y	COHEN. Jitters Jeopardize AIDS Vaccine Trials. Science. 12 November 1993, Vol 262, pages 980-981, see entire document.	6-8, 11, 12
Y	WANG et al. Single Amino Acid Substitution In Constant Region 1 Or 4 Of gp120 Causes The Phenotype of a Human Immunodeficiency Virus Type 1 Variant With Mutations In Hypervariable Regions 1 and 2 To Revert. Journal of Virology. January 1996, Vol 70. No. 1. pages 607-611, see entire document.	1-5, 9, 10
Y	FOX. No Winners Against AIDS. Bio/Technology. February 1994. Vol 12. page 128, see entire document.	6-8, 11, 12